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Epigenetic Therapy of Hematopoietic Malignancies: Novel Approaches for Tissue-Specific and Global Inhibition of EZH2 Enzymatic Activities

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14. ABSTRACT

Direct sequencing of hematopoietic cancers identified gain-of-function mutations of EZH2, the gene encoding the enzymatic subunit of *Polycomb Repressive Complex-2* (*PRC*2), among ~10% germ-center B-cell lymphomas. EZH2 silences gene expression through catalysis of methylation of histone H3 lysine 27. However, the currently available EZH2-specific inhibitors are ineffective for treating EZH2-wildtype lymphomas. Novel therapeutics needs to be developed. We found overexpression of PHF19, a PRC2-associated cofactor, is common among B-cell derived malignancies. During this funding period, we have made significant progress in testing our central hypothesis is that, overexpression of PHF19 confers oncogenicity to lymphoma by either enhancing enzymatic activities or chromatin association of PRC2 complexes; in addition, we have evaluated the pan PRC2 inhibitor as a novel means for blockade of unwanted PRC2 hyperactivities among blood cancers including B-cell malignancies.

15. SUBJECT TERMS
hematopoietic cancer, PRC2, inhibitor, histone methylation

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1. INTRODUCTION:

Direct sequencing of hematopoietic cancers identified gain-of-function mutations of EZH2, the gene encoding the enzymatic subunit of Polycomb Repressive Complex-2 (PRC2), among ~10% germ-center B-cell lymphomas. EZH2 silences gene expression through catalysis of methylation of histone H3 lysine 27. However, the currently available EZH2-specific inhibitors are ineffective for treating EZH2-wildtype lymphomas. Novel therapeutics needs to be developed. We found overexpression of EZH1, an EZH2-ralated enzyme, and PHF19, an EZH2/EZH1-associated cofactor, are far more common among B-cell derived malignancies. Based on these findings, our central hypothesis is that, overexpression of EZH1 and/or PHF19 confers oncogenicity to lymphoma by either enhancing enzymatic activities or chromatin association of PRC2 complexes, and that targeting EZH1 or PHF19 provides a novel means for of blockade of unwanted PRC2 hyperactivities. The proposed experiments are organized along the following Specific Aims:

Aim 1: to develop and evaluate the pan EZH2 and EZH1 inhibitor for treating B-cell lymphomas.

Aim 2: to investigate PHF19 overexpression in conferring EZH2 and EZH1 hyperactivity to lymphoma to promote oncogenesis.

Aim 3: to map the differential binding of EZH2 in B- versus T- cell lineages, and to identify the responsible tissue-specific recruiters.

2. KEYWORDS:

Hematopoietic cancer, PRC2, inhibitor, histone methylation, EZH2, PHF19

3. ACCOMPLISHMENTS:

In the Year 1 funding period, significant progress has been made toward the proposed Specific Aims. There are no significant changes in the project or its overall direction.

- **What were the major goals of the project?**

Below list the major goals of the project as stated in the approved SOW, as well as the actual completion dates or the percentage of completion.

Major Task 1: To Treat various lymphoma cell lines with our pan EZH2 and EZH1 inhibitor in vitro

Subtask 1- To assess the effect of our pan EZH2 and EZH1 inhibitor on tumor cell proliferation in ~30 human cell lines

- targeted completion date (1-2 months; 10/1/2014)
- actual completion dates (% completion): 10/1/2014 (100%)

Subtask 2-To dissect the effect of our pan EZH2 and EZH1 inhibitor on cell cycle progression, tumor cell differentiation, and/or apoptosis

- targeted completion date (1 months; 12/1/2014)
- actual completion dates (% completion): 12/1/2014 (100%)

Subtask 3 – To identify a common "core signature" associated with cellular treatment of our EZH2 and EZH1 inhibitor

- targeted completion date (2-3 months; 2/1/2015)
- actual completion dates (% completion): 2/1/2015 (100%)

Summary of Major Task 1-

- targeted completion date (6 months; 2/1/2015)
- actual completion dates (% completion): 2/1/2015 (100%)

Major Task 2: To treat DLBCL xenograft animal models in vivo with the pan EZH2/EZH1 inhibitor

Subtask 4- perform the pharmacokinetic assay and toxicity evaluation of the pan EZH2/EZH1 inhibitor in animals using different compound administration methods
- targeted completion date (2 months; 2/1/2015)
- actual completion dates (% completion): 4/1/2015 (100%)

Subtask 5- establish tumor xenograft models using human cell lines of lymphoma and myeloma.
- targeted completion date (2 months; 6/1/2015)
- actual completion dates (% completion): 8/1/2015 (100%)

Summary of Major Task 2
- targeted completion date (4 months; 6/1/2015)
- actual completion dates (% completion): 8/1/2015 (100%)

Major Task 3 – to dissect the role of PHF19 overexpression using human B-cell derived malignant tumor lines

Subtask 6- perform gene knockdown of PHF19 followed by assays for tumor cell proliferation using >10 different B-cell derived malignant cell lines
- targeted completion date (1-2 months; 3/1/2015)
- actual completion dates (% completion): 3/1/2015 (100%)

Subtask 7 –assess the effect of knockdown of PHF19 on cell cycle, tumor cell differentiation, and apoptosis
- targeted completion date (1-2 months; 4/1/2015)
- actual completion dates (% completion): 4/1/2015 (100%)

Summary of Major Task 3
- targeted completion date (3 months; 6/1/2015)
- actual completion dates (% completion): 6/1/2015 (100%)

Major Task 4 – to dissect the role of PHF19 overexpression using xenograft models of lymphoma and myeloma

Subtask 8- to establish tumor xenograft models using human B-cell derived malignant cell lines
- targeted completion date (1 months; 7/1/2015)
- actual completion dates (% completion): 8/1/2015 (100%)

- **What was accomplished under these goals?**
  - *For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.*

(1) Major activities
This current progress report covers the following aims as originally proposed:

Aim 1: to develop and evaluate the pan EZH2 and EZH1 inhibitor for treating B-cell lymphomas.
Aim 1.1- to treat DLBCL with our pan EZH2 and EZH1 inhibitor in vitro.

Aim 1.2- to treat DLBCL xenograft animal models in vivo with the pan EZH2/EZH1 inhibitor.

Aim 2: to investigate PHF19 overexpression in conferring EZH2/1 hyperactivity to lymphoma to promote oncogenesis.
Aim 2.1- to dissect the role of PHF19 overexpression using human cell lines and xenograft models of lymphoma and myeloma.

The major activities include:

Major activity 1- To assess the effect of our pan EZH2 and EZH1 inhibitor on tumor cell proliferation in various human tumor cell lines
Major activity 2-To dissect the effect of our pan EZH2 and EZH1 inhibitor on cell cycle progression, tumor cell differentiation, and/or apoptosis
Major activity 3 – To identify a common "core signature" associated with cellular treatment of our EZH2 and EZH1 inhibitor
Major activity 4- perform the pharmacokinetic assay and toxicity evaluation of the pan EZH2/EZH1 inhibitor in animals using different compound administration methods
Major activity 5- establish tumor xenograft models using human cell lines of lymphoma and myeloma.
Major activity 6- perform gene knockdown of PHF19 followed by assays for tumor cell proliferation using >10 different B-cell derived malignant cell lines
Major activity 7 assess the effect of knockdown of PHF19 on cell cycle, tumor cell differentiation, and apoptosis
Major activity 8- establish tumor xenograft models using human B-cell derived malignant cell lines

(2) Specific objectives

2.1- to develop and assess the biological effects by a pan EZH2 and EZH1 inhibitor.

2.2- to demonstrate that the pan EZH2 and EZH1 inhibitor represents a new means for achieving complete inhibition of EZH family enzymes in treating blood cancers.

2.3- to establish PHF19 as a new drug target of B-cell tumors.

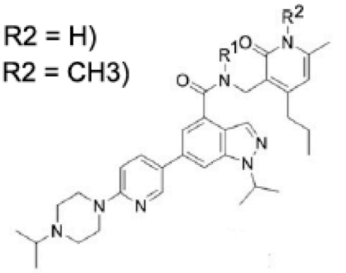
(3) Significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative);

(3.1) Related to Major activity 1- To assess the effect of our pan EZH2 and EZH1 inhibitor on tumor cell proliferation in various human tumor cell lines

- we have identified a set of small-molecule inhibitors for specific targeting of both EZH2 and EZH1, including UNC1999, an EZH2 and EZH1 dual inhibitor, and UNC2400, an inactive analog compound useful for assessment of off-target effect (Fig 1A-B below; also refer to Appendix – Xu B et al

A

UNC1999 (R1, R2 = H)
UNC2400 (R1, R2 = CH3)



B

Compound	R1	R2	IC50 (nM)
UNC1999	H	H	<10
UNC1756	CH_3	H	72
UNC3142	H	CH_3	103
UNC2400	CH_3	CH_3	>13000

Fig 1. A small-molecule UNC1999, and not its inactive analog UNC2400, selectively and potently suppresses H3K27 methylation. (A) Chemical structure of UNC1999 and UNC2400, with the positions R1 and R2 modified with 2 N-methyl groups (CH3) in UNC2400. (B) Summary of modification at R1 and R2 in UNC1999 and derivatives, and their IC50 measured by in vitro methyltransferase assay.

Blood 2015, Fig 1). The discovery of compounds was made with a structure based chemical compound design in a close collaboration with UNC Center for Drug Discovery and Chemical Biology.

- We characterized molecular and cellular effects by these translational tools and aim to establish novel therapeutics for cancer cells. Specifically we show that UNC1999, and not UNC2400, specifically suppressed H3K27me3/2, the enzymatic product of EZH2/1 (Fig 2; also refer to Appendix – Xu B et al Blood 2015, Fig 1). We also have shown that UNC1999 concurrently elevated the cellular level of H3K27ac, while having negligible effects on other histone methylations (Fig 2).

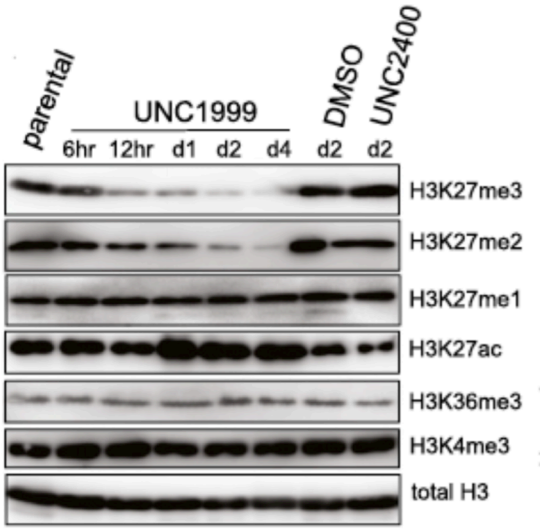
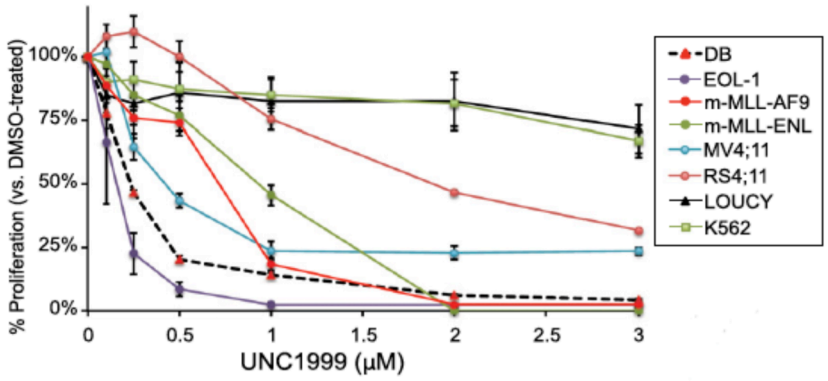


Fig 2. Immunoblot of the indicated histone modifications after treatment with DMSO, or 3 μM UNC1999 or UNC2400.

- Next, we have applied UNC1999 to a larger panel of leukemia cell lines. Many showed sensitivity to UNC1999 (Figure 3; also refer to Appendix – Xu B et al Blood 2015, Fig 2). From the compound treatments shown in Fig 3 (left), we were able to measure the EC50 of UNC1999 in suppressing tumor cell proliferation (Figure 3, right table).



Cell Line	EC50 (μM)	Known Genetic Mutation
DB	0.227	*EZH2 Y641N*
EOL-1	0.1019	*MLL-PTD; FIP1L1-PDGFRA*
K562	N/A (>250)	*BCR-ABL*
LOUCY	N/A (>200)	*EZH2 deleted*
m-MLL-AF9 (4 lines)	0.404~0.621	*MLL-AF9*
m-MLL-ENL (2 lines)	0.547~0.836	*MLL-ENL*
MV4;11	0.5248	*MLL-AF4*
OCI-AML2	1.5	*MLL-AF9*
RS4;11	1.956	*MLL-AF4*

Fig 3. Relative proliferation of a panel of leukemia or lymphoma cell lines treated with various concentrations of UNC1999 for 16 days (left panel). Y-axis, presented as the mean of triplicates 6 SD, represents the relative percentage of accumulative cell numbers after normalization to DMSO treatment. Shown as a dashed line is DB, an EZH2-mutated (Y641N) lymphoma line known to be sensitive to EZH2 inhibition. Right panels show summary of EC50 of a panel of cell lines in response to UNC1999.

Major findings & conclusion

- UNC1999 induces potent and selective suppression of H3K27me3/2, whereas UNC2400 does not, highlighting them as a pair of compounds useful to manipulate both PRC2-EZH2 and PRC2-EZH1.
- Collectively, we show that our lead compound UNC1999, an EZH2 and EZH1 dual inhibitor, efficiently suppresses proliferation of blood cancer cells that co-express EZH2 and EZH1.

(3.2) Related to Major activity 2-To dissect the effect of our pan EZH2 and EZH1 inhibitor on cell cycle progression, tumor cell differentiation, and/or apoptosis

- We have shown that UNC1999 induced cell-cycle arrest at the G1-to-S transition (Figure 4, right; also refer to Appendix – Xu B et al Blood 2015, Fig 5). In contrast, UNC2400 did not alter

cell-cycle progression (Figure 4, middle vs. left). By using EOL1 cells as a working model, we have also studied the effect of our pan EZH2 and EZH1 inhibitor on cancer cell apoptosis (Figure 5, right; also refer to Appendix – Xu B et al Blood 2015, Fig 3). We found a time- and concentration dependent induction of apoptosis and cell viability after treatment with UNC1999, and not UNC2400 (Figure 5).

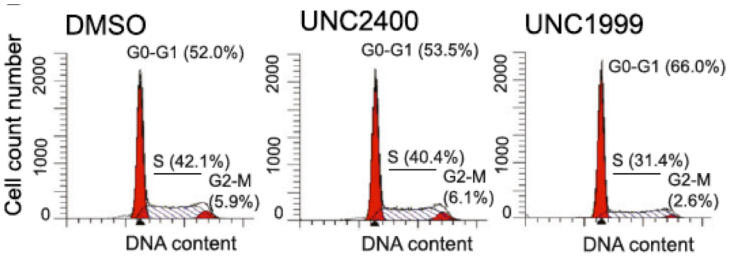


Fig 4. Representative histograms showing DNA contents measured by PI staining of leukemia cells after treatment with 3 μM of compounds for 2 days.

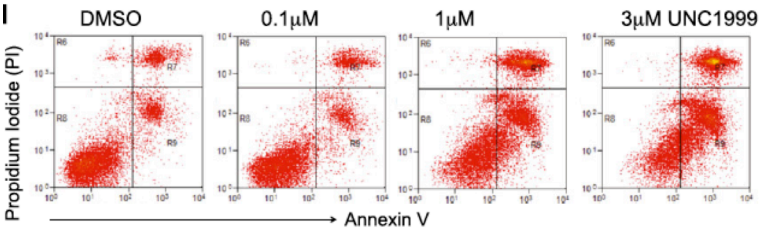


Fig 5. Typical profiles of staining with PI and annexin V after treatment of EOL-1 cells with DMSO or the indicated concentration of UNC1999 for 6 days. PI, propidium iodide.

Major findings & conclusion-

- UNC1999, but not UNC2400, suppresses growth of malignant blood tumor cells by inhibiting cell cycle progression.
- UNC1999, but not UNC2400, suppresses growth of leukemia cells by promoting apoptosis of blood cancer cells.

(3.3) Related to Major activity 3 – To identify a common "core signature" associated with cellular treatment of our EZH2 and EZH1 inhibitor

- To dissect the underlying mechanisms for the UNC1999-induced anti-leukemia effect, we have carried out gene transcriptome profilings and aimed to identifying a common "core signature" associated with cellular treatment of our EZH2 and EZH1 inhibitor. These analyses were carried out in UNC genomic core using an Affymetrix gene-array platform.
- We found that UNC1999 altered the expression of a few hundred transcripts (Figure 6A-B; also refer to Appendix – Xu B et al Blood 2015, Fig 4), and consistent with the silencing role of PRC2, significantly more genes showed upregulation than downregulation after UNC1999 treatment (Figure 6A-B). In contrast, UNC2400 induced little changes.
- Importantly, the transcripts upregulated by UNC1999 largely overlapped those after knockdown of EED, a common cofactor ofEZH2 andEZH1, demonstrating on-target effects of UNC1999 (Figure 6D). GSEA revealed significant enrichment of PRC2-repressed genes (Figure 6E-G) and those associated with differentiation (Figure 6H) in UNC1999- vs DMSO-treated cells.
- By RT-qPCR, we verified the gene expression changes following UNC1999 vs UNC2400 treatments or after EED knockdown (Figure 6I).

Major findings & conclusion-

- Taken together, our data have demonstrated that treatment of blood cancer cells with UNC1999, an EZH2 and EZH1 dual inhibitor, specifically derepresses their PRC2 gene targets.
- UNC2400, an inactive analog of UNC1999, has little effects on gene expression programs.

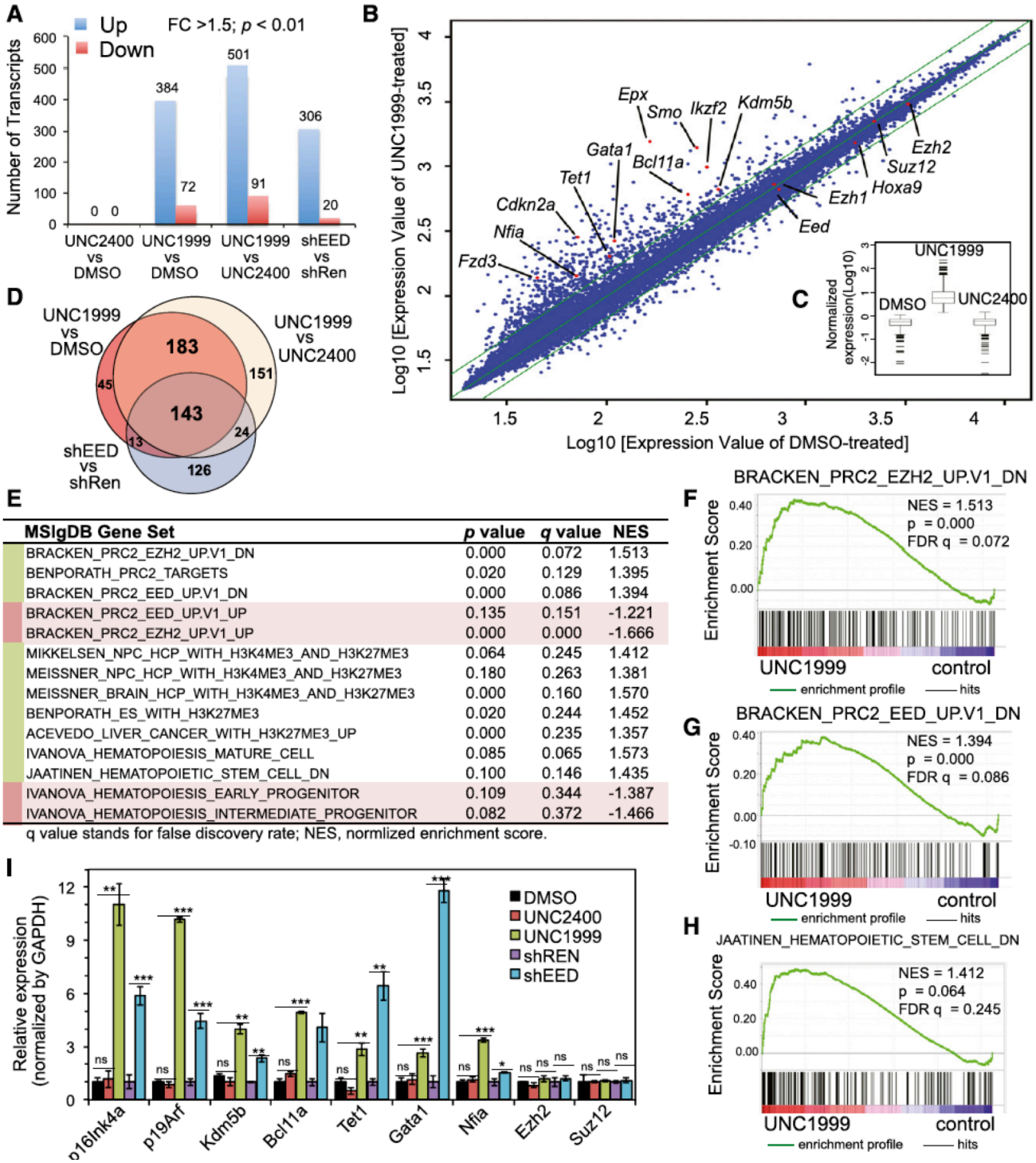


Fig 6. UNC1999, and not UNC2400, derepresses the PRC2 gene targets. (A) Summary of the upregulated (blue) and downregulated (red) transcripts after a 5-day treatment with 3 μM of compounds or after knockdown of EED vs Renilla, as identified by microarray analysis with a cutoff of FC of 1.5 and a P value of 0.01. (B) Scatter plot to compare the global gene expression pattern in MLL-AF9–transformed leukemia cells following DMSO (x-axis) vs UNC1999 treatment (y-axis). Plotted are Log10 values of the signal intensities of all transcripts on gene microarrays after normalization. The flanking lines in green indicate 1.5-fold change in gene expression. (C) Boxplots showing the expression levels of upregulated transcripts in the compound- vs DMSO-treated samples. (D) Venn diagram of the upregulated transcripts shown in panel A. (E) Summary of GSEA using the MSIgDB. Green and red indicate the positive and negative correlation to UNC1999-treated cells, respectively. (F-H) GSEA revealing significant enrichment of the EZH2-repressed (F) or EED repressed gene signatures (G) and those negatively associated with hematopoietic stem cells (H) in the UNC1999- vs DMSO-treated cells. (I) RT-qPCR detects relative expression levels of the indicated genes following treatment with compounds or EED knockdown (shEED) for 5 days. Y-axis represents fold-change after normalization to GAPDH and to control (DMSO treatment or Renilla knockdown [shRen]).

(3.4) Related to Major activity 4 & 5 – to perform the pharmacokinetic assay and toxicity evaluation of the pan EZH2/EZH1 inhibitor in animals using different compound administration methods; to establish tumor xenograft models using human cell lines of lymphoma and myeloma.

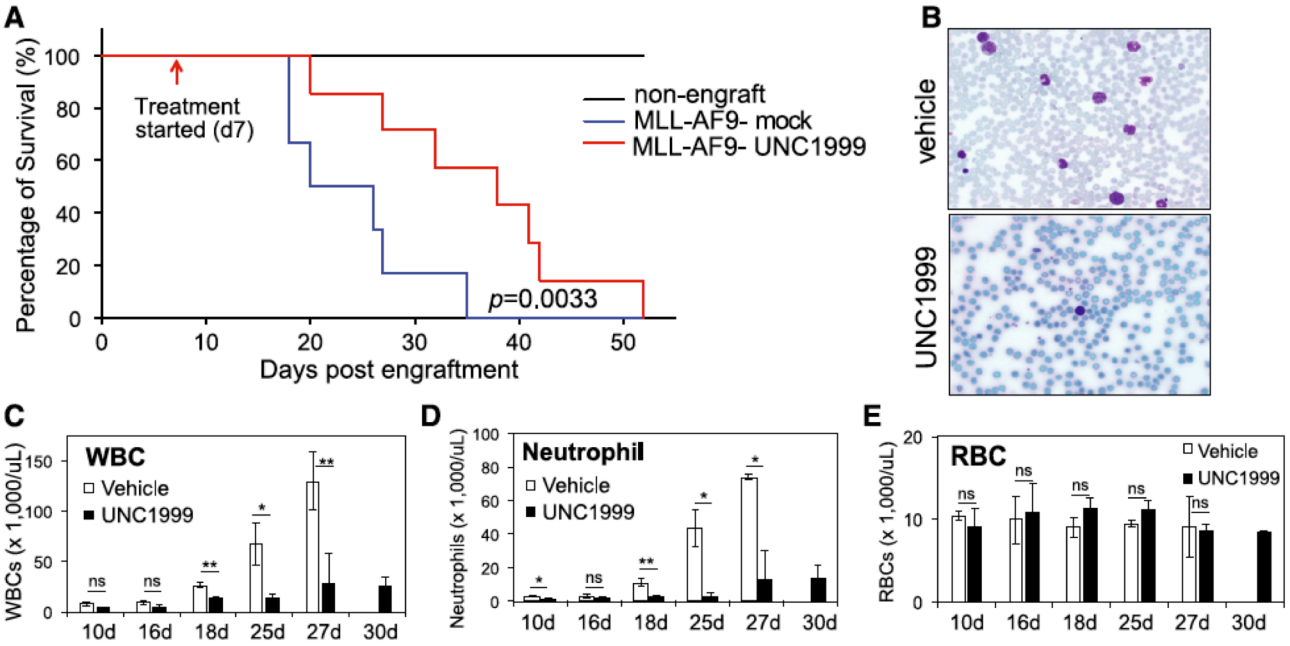


Fig 7- UNC1999 prolongs survival of animal leukemia models in vivo. (A) Kaplan-Meier curve showing disease kinetics. Mice received oral administration of either vehicle (blue) or 50 mg/kg UNC1999 (red) twice per day. Black lines (top) represent nontransplanted normal mice treated with vehicle or UNC1999. Cohort size, 6 to 7 mice. (B) Typical Wright-Giemsa staining images of the peripheral blood smears prepared from the vehicle- (top) and UNC1999-treated (bottom) leukemia mice 25 days post cancer induction. (C-E) Summary of counts of the WBCs (C), neutrophils (D), and RBCs (E) in the peripheral blood of vehicle- (white) or UNC1999-treated (black) leukemia mice at the indicated date post cancer induction.

- To examine the effect of UNC1999 on in vivo leukemogenesis, we developed a treatment regimen by administering either vehicle or 50 mg/kg UNC1999 by oral gavage to mice twice per day. We show that UNC1999 treatment did not affect overall survival of normal mice (Fig 7A, black line), or the counts of red blood cells or platelets (Figure 7C). These findings indicate lack of toxicity associated with the treatment of our pan EZH2/EZH1 inhibitor, UNC1999, in live animals.
- We then monitored cancer progression by periodic assessment of peripheral blood samples and found that, despite steady accumulation of WBCs in both vehicle- and UNC1999-treated cohorts, UNC1999-treated animals displayed significant reduction in WBC counts (Figure 7B-D), indicating a delayed leukemic progression. Indeed, UNC1999-treated leukemic mice exhibited a significantly prolonged survival, with a latency of 36.6 6 11.7 days in contrast to 24 6 6.7 days for vehicle-treated ones (Fig 7A, red versus blue lines).

Major findings & conclusion-

Collectively, these above data show that

- Our established protocol for oral administration of UNC1999 does not cause obvious toxicity in tested animals;
- Oral delivery of UNC1999 delays leukemogenicity in vivo and our EZH2 and EZH1 dual

inhibitor provides a new therapeutics for blood malignancies.

(3.5) Related to Major activity 6 to 8 -

- Knockdown (KD) of *PHF19* in multiple B-cell derived malignant tumor lines revealed that *PHF19* is required for *in vitro* tumor growth (Fig. 8A). The *in vitro* phenotypes of *PHF19* KD can be rescued by re-introduction of PHF19 (Fig 8A, red).
- Using NCI-H929 cells, a widely-used B-cell tumor line, we show that *PHF19* KD dramatically impairs the tumor-sphere formation *in vitro* (Fig. 8B). We also found loss of PHF19 inhibits cell cycle progression and promotes apoptosis.

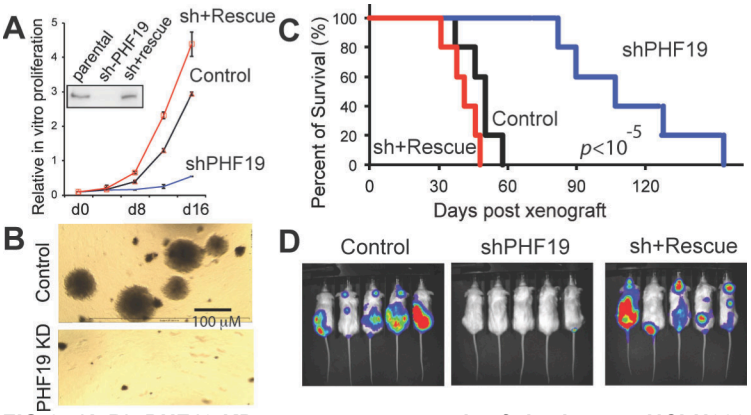


FIG 8. (A-B) *PHF19* KD suppresses growth of the human NCI-H929 multiple myeloma (MM) cells *in vitro* (A) and dramatically impairs the tumor sphere-forming capacity in the soft agar assay (B). (C) Kaplan-Meier plots showing a crucial role of *PHF19* in *in vivo* growth of tumor induced by inoculation of the NCI-H929 cells, following *PHF19* KD (blue) and rescue (red) in comparison to mock (black). (D) Live bioluminescence imaging of MM xenografts (labeled with a luciferase reporter) 6 weeks post inoculation.

- we have used the SCID-NOD-gamma (SNG) mice which were xenografted intravenously with human multiple myeloma (a type of plasma B cell tumor) cell lines (Fig 8, black); we found that knockdown (KD) of *PHF19* delays the xenografted tumor formation *in vivo* (Fig. 8C-D). The *in vivo* phenotypes of *PHF19* KD can be rescued by re-introduction of *PHF19* (Fig 8C, red; Fig 8D, right panel).

Major findings & conclusion-

-- Clinical relevance – we found that PHF19 is among the top overexpressed genes in lymphoma and multiple myeloma (MM). In addition, there is a steady increased expression level of PHF19 mRNA among multiple myeloma (MM) and plasma cell leukemia (PCL), in comparison to normal plasma cells.
- PHF19 is crucial for tumor cell proliferation in tested B-cell derived malignant cell lines
- we have established the tumor xenograft models (Fig 8D) using human B-cell derived malignant cell lines

(4) other achievements-

In addition, I have been invited to write timely review articles on the topic related to the proposed research, which have been published on Wang GG et al Blood 2015 and Xu B et al Exp Hematology 2015 (also refer to Appendix).

- **What opportunities for training and professional development has the project provided?**

Training and professional development provided to Dr. Zhihong Ren MD/PHD, who worked on the project as a postdoc researcher-

"Training" activities:

- xenograft studies with human cell lines and SCID nude mice models
- live imaging
- in-house postdoc seminar (weekly)
- workshop of cancer research using laboratory animals

- **How were the results disseminated to communities of interest?**

The outcomes of the proposed research (year 1), specifically one research article (Xu B et al Blood 2015) and two review articles (Wang GG et al Blood 2015 and Xu B et al Exp Hematology 2015; also refer to Appendix), were deposited to the publicly available portal or databases such as PubMed Central. These research results were downloaded and viewed by numerous researchers and clinicians over the world. Since their publications we have received many emails commenting possible implications of our research results. These three published works have been cited over a dozen of times.

What do you plan to do during the next reporting period to accomplish the goals?

In the second funding year, I hope to publish additional papers based on observations and data obtained for Specific Aim #1-2. Despite a delay due to annual renewal of an expired animal protocol (see below section 5), I will continue to generate the data as mentioned in the original proposal and timeline table. Since significant progress was made toward all proposed specific aims, no important modifications have been made the original plans at this point.

4. IMPACT:

- **What was the impact on the development of the principal discipline(s) of the project?**

The findings and research results are likely to make an impact on blood cancer research and therapies in the following ways:

  - Define a set of new 'Achilles' heels' of blood cell derived malignancies.
  - Targeting these new drug targets with inhibitors we develop shall provide novel therapeutic interventions.

- **What was the impact on other disciplines?**

Nothing to Report.

- **What was the impact on technology transfer?**

Nothing to Report.

- **What was the impact on society beyond science and technology?**

Nothing to Report.

5. CHANGES/PROBLEMS:

- **Changes in approach and reasons for change**

Nothing to Report.

- **Actual or anticipated problems or delays and actions or plans to resolve them**

Delays encountered during the reporting period and actions or plans to resolve them:
Due to an expired animal protocol and its renewal, we had to stop any animal related research or work relating to this funding. The delay was from 2/23/2015- 5/21/2015.
We will continue to pursue the proposed aims during the 2nd year funding phase.

- **Changes that had a significant impact on expenditures**

Nothing to Report.

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

Nothing to Report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

Xu B, On DM, Ma A, Parton T, Konze KD, Pattenden SG, Allison DF, Cai L, Rockowitz S, Liu S, Liu Y, Li F, Vedadi M, Frye SV, Garcia BA, Zheng D, Jin J, **Wang GG**. Selective inhibition of EZH2 and EZH1 enzymatic activity by a small molecule suppresses MLL-rearranged leukemia. **Blood.** 2015 Jan 8; 125(2):346-57. PMCID: PMC4287641.

-acknowledgement of federal support (yes).

Wang GG, Konze KD, Tao J. Polycomb genes, miRNA, and their deregulation in B-cell malignancies. **Blood**. 2015 Feb 19;125(8):1217-1225. PMCID: PMC4335077.

- -acknowledgement of federal support (yes).

Xu B, Konze KD, Jin J, **Wang GG**. Targeting EZH2 and PRC2 dependence as novel anti-cancer therapy. **Exp Hematol**. 2015 May 28. pii: S0301-472X(15)00163-0. doi:10.1016/ j.exphem.2015.05.001. [Epub ahead of print]

-acknowledgement of federal support (yes).

Books or other non-periodical, one-time publications.

Nothing to Report.

Other publications, conference papers, and presentations.

Nothing to Report.

Website(s) or other Internet site(s)

Nothing to Report

Technologies or techniques

Nothing to Report

Inventions, patent applications, and/or licenses

Nothing to Report

Other Products

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	*Gang (Greg) Wang, PHD & Assistant Professor*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	**orcid.org/0000-0002-7210-9940**
Nearest person month worked:	*5*
Contribution to Project:	*Dr. Wang has served as team leader performing experimental design, guidance and data review/interpretation.*
Funding Support:	*NIH, Kimmel Foundation*

▪

Name:	*Zhihong Ren, PHD/MD (postdoc trainee)*
Project Role:	*postdoc*
Researcher Identifier (e.g. ORCID ID):	**n/a**
Nearest person month worked:	*10*
Contribution to Project:	*Dr. Ren has served as postdoc research performing in vitro and in vivo experiments as proposed; he also carried out data review/interpretation.*
Funding Support:	*n/a*

▪

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report

What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

N/A

9. **APPENDICES –**three **Journal publications (see attached below)**

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Regular Article

MYELOID NEOPLASIA

Selective inhibition of EZH2 and EZH1 enzymatic activity by a small molecule suppresses *MLL*-rearranged leukemia

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Key Points

- We characterize active vs inactive analog compounds suitable for inhibition of both PRC2-EZH2 and PRC2-EZH1 ex vivo and in vivo.
- This study is the first to show oral delivery of an EZH2 and EZH1 dual inhibitor as promising therapeutics for *MLL*-rearranged leukemia.

Enhancer of zeste homolog 2 (EZH2) and related EZH1 control gene expression and promote tumorigenesis via methylating histone H3 at lysine 27 (H3K27). These methyltransferases are ideal therapeutic targets due to their frequent hyperactive mutations and overexpression found in cancer, including hematopoietic malignancies. Here, we characterized a set of small molecules that allow pharmacologic manipulation of EZH2 and EZH1, which include UNC1999, a selective inhibitor of both enzymes, and UNC2400, an inactive analog compound useful for assessment of off-target effect. UNC1999 suppresses global H3K27 trimethylation/dimethylation (H3K27me3/2) and inhibits growth of mixed lineage leukemia (*MLL*)–rearranged leukemia cells. UNC1999-induced transcriptome alterations overlap those following knockdown of embryonic ectoderm development, a common cofactor of EZH2 and EZH1, demonstrating UNC1999's on-target inhibition. Mechanistically, UNC1999 preferentially affects distal regulatory elements such as enhancers, leading to derepression of polycomb targets including *Cdkn2a*. Gene derepression correlates with a decrease in H3K27me3 and concurrent gain in H3K27 acetylation. UNC2400 does not induce such effects. Oral administration of UNC1999 prolongs survival of a well-defined murine leukemia model bearing *MLL-AF9*. Collectively, our study provides the detailed profiling for a set of chemicals to manipulate EZH2 and EZH1 and establishes specific enzymatic inhibition of polycomb repressive complex 2 (PRC2)–EZH2 and PRC2-EZH1 by small-molecule compounds as a novel therapeutics for *MLL*-rearranged leukemia. (*Blood*. 2015;125(2):346-357)

Introduction

Covalent histone modification provides a fundamental means to control gene expression and define cellular identities.[1-3] Dysregulation of histone modification represents a central oncogenic pathway in human cancers.[1,3,4] As the regulatory factors involved in the installation, removal, or recognition of histone modification (often termed as epigenetic "writers," "erasers," and "readers"[1]) are increasingly considered to be "druggable,"[5-7] development of epigenetic modulators holds promise for novel therapeutic interventions.[7,8]

Polycomb repressive complex 2 (PRC2), the sole enzymatic machinery that uses either enhancer of zeste homolog 2 (EZH2) or related EZH1 as a catalytic subunit to induce trimethylation of histone H3 at lysine 27 (H3K27me3), has been shown to play critical roles in gene silencing[9] and in hematopoietic lineage specification at various developmental stages.[10-13] Extensive evidence has linked PRC2 deregulation to malignant hematopoiesis. Recurrent *EZH2* gain-of-function mutations were found in germinal center B-cell lymphoma patients,[14,15] and constitutive expression of wild-type or lymphoma-associated mutant *EZH2* in hematopoietic lineages induced myeloproliferative diseases[16] and lymphomagenesis,[13,17] respectively, in murine models. Furthermore, EZH1 compensates the function of EZH2[9,18] and emerges as regulator of myeloid neoplasms.[19,20] Inhibitors selective to EZH2 have recently been developed and shown to be effective in killing lymphoma cells with *EZH2* mutation,[21-23] however, these inhibitors demonstrated minimal effects on proliferation or gene transcription among lymphomas carrying the wild-type EZH2[21,22,24] and are expected to be ineffective for tumors that rely on both wild-type EZH2 and EZH1.

Submitted June 9, 2014; accepted November 3, 2014. Prepublished online as *Blood* First Edition paper, November 13, 2014; DOI 10.1182/blood-2014-06-581082.

B.X., D.M.O., and A.M. contributed equally to this study.

The ChIP-seq data reported in this article have been deposited in the Gene Expression Omnibus database (accession number GSE62437). The microarray data reported in this article have been deposited in the Gene Expression Omnibus database (accession number GSE62198).

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

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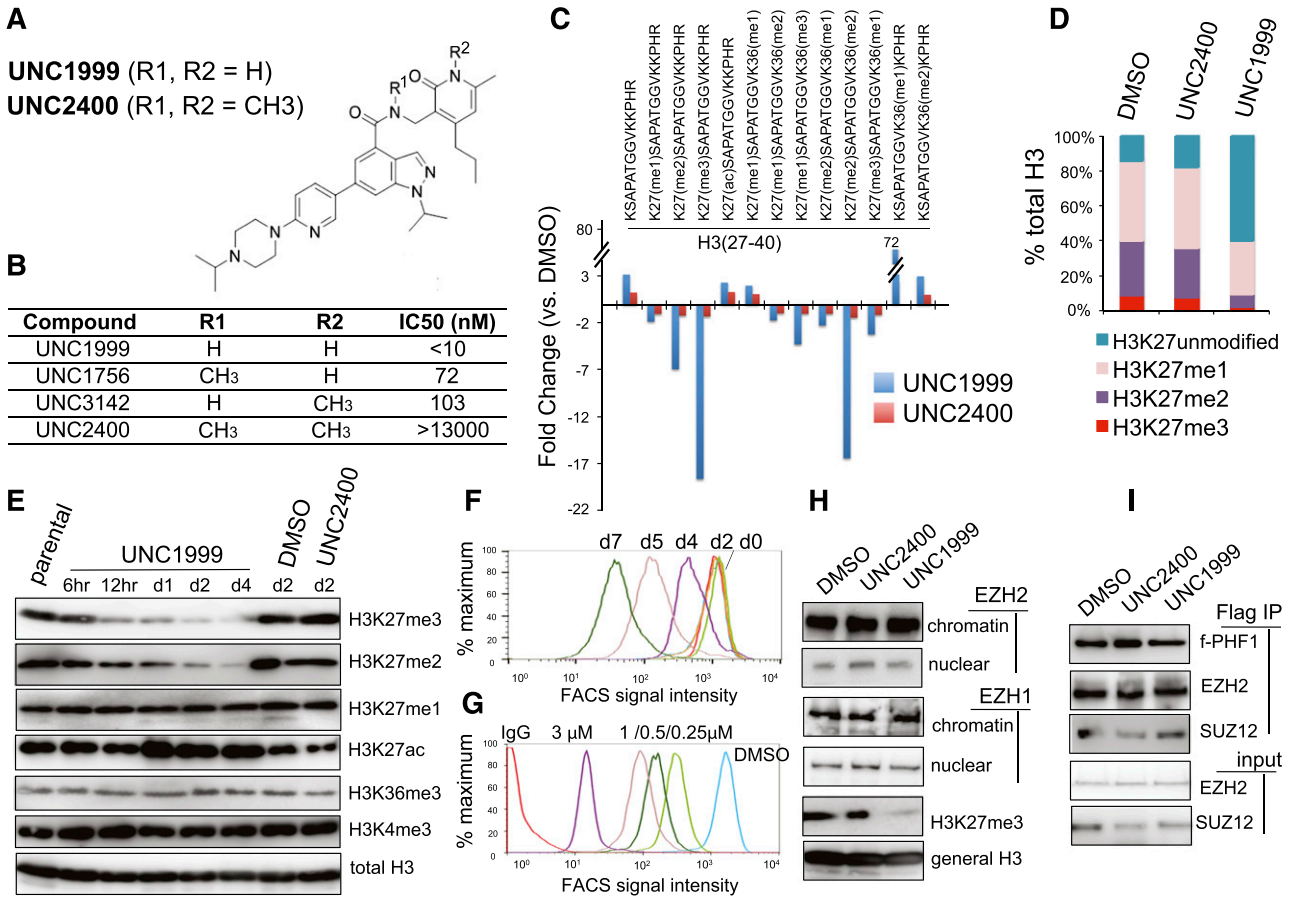

Compound	R1	R2	IC50 (nM)
UNC1999	H	H	<10
UNC1756	CH_3	H	72
UNC3142	H	CH_3	103
UNC2400	CH_3	CH_3	>13000

Figure 1. A small-molecule UNC1999, and not its inactive analog UNC2400, selectively and potently suppresses H3K27 methylation. (A) Chemical structure of UNC1999 and UNC2400, with the positions R1 and R2 modified with 2 N-methyl groups (CH_3) in UNC2400. (B) Summary of modification at R1 and R2 in UNC1999 and derivatives, and their IC_{50} measured by in vitro methyltransferase assay. (C) Quantitative mass spectrometry analysis detects the change in relative abundance of various peptide species covering histone H3 amino acids 27-40 after treatment with 3 μM UNC1999 (blue) or UNC2400 (red) for 4 days. Y-axis represents fold-change in relative abundances normalized to DMSO-treated samples; the sequence and modification of H3 peptide are shown on top. (D) Overall percentages of histone H3 with the lysine 27 either unmodified, monomethylated, dimethylated, or trimethylated (H3K27me1/2/3) following compound treatments. (E) Immunoblot of the indicated histone modifications in *MLL-AF9*–transformed leukemia progenitor cells after treatment with DMSO, or 3 μM UNC1999 or UNC2400. (F-G) Flow cytometry with H3K27me3-specific antibodies revealing time-dependent (F, 2 μM UNC1999) and dose-dependent suppression of H3K27me3 by UNC1999 (G, 7-day treatment) in *MLL-AF9*–transformed murine leukemia cells and *EOL-1* human leukemia cells, respectively. DMSO and nonspecific IgG are used as control. (H) Immunoblots detecting the chromatin-bound and nucleoplasmic fraction of EZH2 or EZH1 after treatment with 2 μM of the indicated compounds for 5 days. (I) Co-IP of PRC2 complex components following Flag IP with extracts of a Flag-PHF1 stable expression cell line[34] in the presence of 2 μM of the indicated compounds. ac, acetylation; Co-IP, coimmunoprecipitation; FACS, fluorescence-activated cell sorter; Ig, immunoglobulin; IP, immunoprecipitation; me1/2/3, mono/di/trimethylation.

Recently, we have discovered a series of small-molecule compounds for specific targeting of both EZH2 and EZH1, including UNC1999, an EZH2 and EZH1 dual inhibitor, and UNC2400, an inactive analog compound useful for assessment of off-target effect.[25] Here, we characterized molecular and cellular effects by these translational tools and aim to establish novel therapeutics for cancer types that rely on PRC2-EZH2 and PRC2-EZH1 both. We choose to focus on leukemia bearing chromosomal rearrangement of mixed lineage leukemia (*MLL*), a gene encoding histone H3 lysine 4 (H3K4)–specific methyltransferase.[1,26] *MLL* rearrangements are responsible for ~70% of infant acute myeloid or lymphoid leukemia and ~7% to 10% of adult cases,[26] and leukemia with *MLL* rearrangement displays poor prognosis with low survival rates, highlighting a special need for new interventions.[27,28] Oncoproteins produced by *MLL* rearrangements inappropriately recruit epigenetic factors and/or transcriptional elongation machineries to enforce abnormal gene expression.[1,26-28] Recent studies show that PRC2 acts in parallel with *MLL* rearrangements by controlling a distinctive gene program to sustain leukemogenicity.[19,20,29] Specifically, EZH2 and EZH1 compensate one another to promote acute leukemogenesis, and genetic disruption of both enzymes was required to inhibit growth of leukemia carrying *MLL-AF9*, a common form of *MLL* rearrangements.[19,20] Therefore, chemical agents that can target both PRC2-EZH2 and PRC2-EZH1 shall represent a new way for treating *MLL*-rearranged leukemia.

In this study, we use a series of proteomics, genomics, and tumorigenic assays to profile the effects of our unique EZH2 and EZH1 dual inhibitor, UNC1999, and its inactive analog, UNC2400, among *MLL*-rearranged leukemia. UNC1999, and not UNC2400, specifically suppressed H3K27me3/2 and induced a range of anti-leukemia effects including anti-proliferation, differentiation, and apoptosis. The UNC1999-responsive gene signatures include *Cdkn2a* and developmental genes, and significantly overlapped those induced by knockdown of EED, an essential subunit of PRC2-EZH2 and PRC2-EZH1. Mechanistically, we unveiled preferential "erasure" of H3K27me3 associated with distal regulatory elements such as enhancers following UNC1999 treatment, whereas H3K27me3 peaks at proximal promoters are largely retained,

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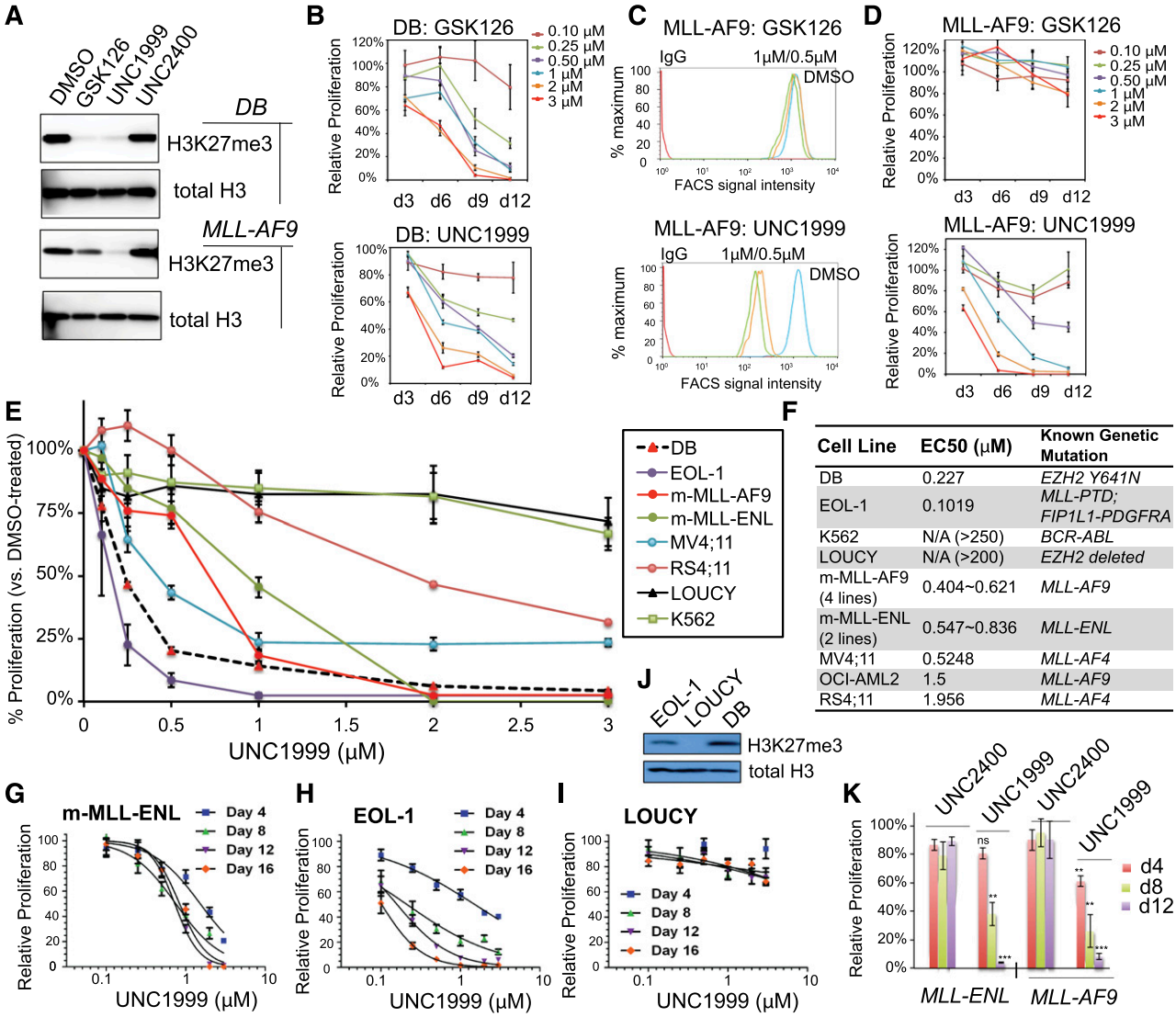

Cell Line	EC50 (μM)	Known Genetic Mutation
DB	0.227	*EZH2 Y641N*
EOL-1	0.1019	*MLL-PTD; FIP1L1-PDGFRA*
K562	N/A (>250)	*BCR-ABL*
LOUCY	N/A (>200)	*EZH2 deleted*
m-MLL-AF9 (4 lines)	0.404~0.621	*MLL-AF9*
m-MLL-ENL (2 lines)	0.547~0.836	*MLL-ENL*
MV4;11	0.5248	*MLL-AF4*
OCI-AML2	1.5	*MLL-AF9*
RS4;11	1.956	*MLL-AF4*

Figure 2. UNC1999, but not GSK126, efficiently suppresses H3K27me3 in *MLL*-rearranged leukemia cells and inhibits their growth. (A) Immunoblots of the global H3K27me3 level after treatment of *DB* lymphoma cells (top) or *MLL-AF9*–transformed murine leukemia progenitors (bottom) with 2 μM of the indicated compounds for 5 days. General H3 serves as control. (B) Relative proliferation of *DB* cells treated with a range of concentrations of GSK126 (top) or UNC1999 (bottom) for the indicated duration. Y-axis represents the relative percentage of accumulative cell numbers normalized to DMSO treatment, and is presented as the mean of triplicates ± SD. (C) Flow cytometry analysis of H3K27me3 in *MLL-AF9*–transformed murine leukemia progenitors following treatment with various concentrations of GSK126 (top) or UNC1999 (bottom) for 4 days. DMSO serves as control. (D) Relative proliferation of *MLL-AF9*–transformed leukemia progenitors treated with a range of concentrations of GSK126 (top) or UNC1999 (bottom) for the indicated duration. Y-axis represents the relative percentage of cell numbers after normalization to DMSO treatment, and is presented as the mean of triplicates ± SD. (E) Relative proliferation of a panel of leukemia or lymphoma cell lines treated with various concentrations of UNC1999 for 16 days. Y-axis, presented as the mean of triplicates ± SD, represents the relative percentage of accumulative cell numbers after normalization to DMSO treatment. Shown as a dashed line is *DB*, an *EZH2*-mutated (Y641N) lymphoma line known to be sensitive to EZH2 inhibition.[22] (F) Summary of EC_{50} of a panel of cell lines in response to UNC1999. m-MLL-AF9 and m-MLL-ENL represent murine leukemia lines established by *MLL-AF9* and *MLL-ENL*, respectively. (G-I) Relative proliferation of murine *MLL-ENL*–bearing leukemia cells (G) and *EOL-1* (H) and *LOUCY* (I) human leukemia cells treated with a range of UNC1999 concentrations for the indicated duration. Y-axis represents relative percentage of accumulative cell numbers after normalization to DMSO treatment, and is presented as the mean of triplicates ± SD. (J) Immunoblot of H3K27me3 and general H3 in *EOL-1*, *LOUCY*, and *DB* cells. (K) Relative proliferation of murine leukemia cells bearing *MLL-AF9* or *MLL-ENL* after treatment with UNC1999 or UNC2400 and normalization to DMSO treatment. **P* < .05; ***P* < .01; ****P* < .001; ns, not significant.

despite a shrinking in their average peak size. Gene derepression correlates with decrease in H3K27me3 and concurrent gain in H3K27 acetylation (H3K27ac). None of these effects were seen following UNC2400 treatment, further verifying on-target effect by UNC1999. *Cdkn2a* is a crucial mediator for UNC1999-induced growth inhibition. Importantly, oral dosing of UNC1999 prolongs survival of *MLL-AF9*–induced murine leukemia models. Thus, our study provides a detailed characterization of a pair of small-molecule compounds available to the community for studying EZH2 and EZH1 in health and disease. This study also represents the first one to establish chemical inhibition of both EZH2 and EZH1 as a promising therapeutics for *MLL*-rearranged leukemia.

Methods

Compound synthesis and usage

UNC1999 and UNC2400 were synthesized as previously described.[25] Synthesis of UNC1756 and UNC3142 is described in supplemental Materials

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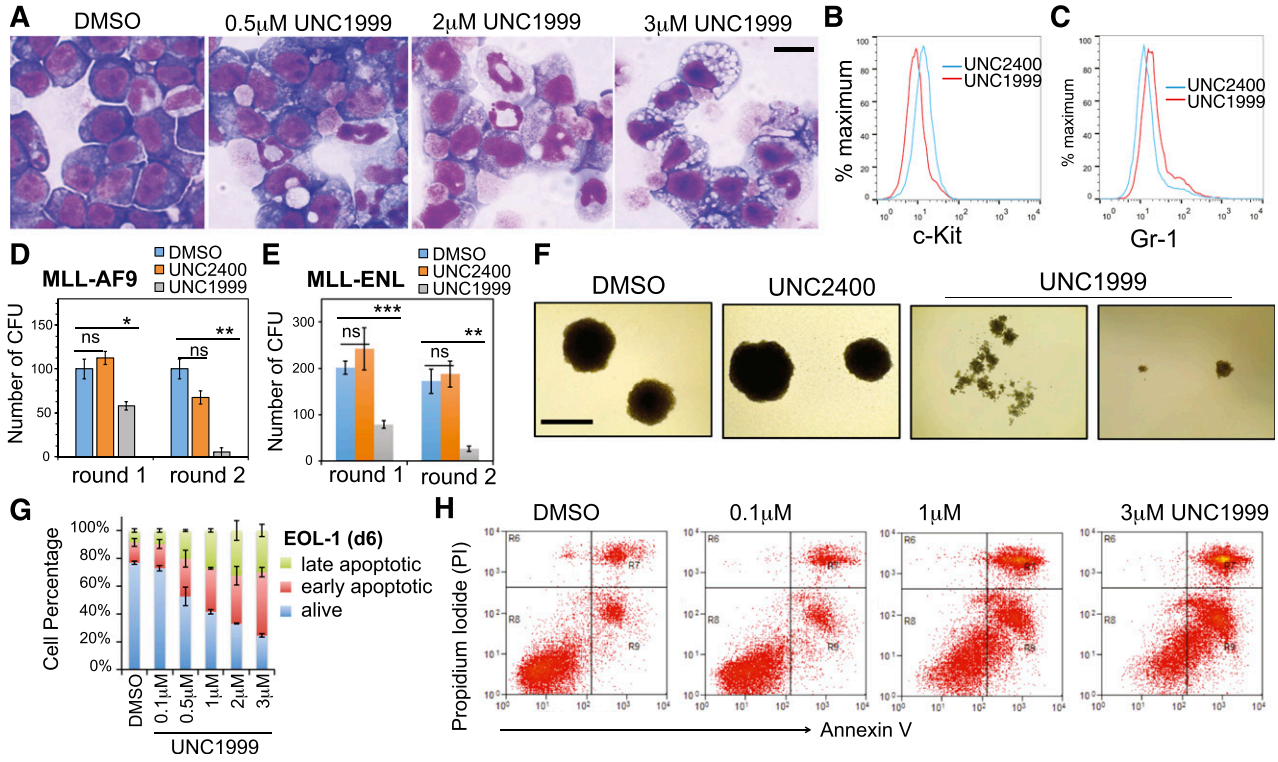

Figure 3. UNC1999, and not UNC2400, promotes differentiation, suppresses colony formation, and induces apoptosis of *MLL*-rearranged leukemia cells. (A) Representative light micrographs show Wright-Giemsa staining of *MLL-AF9*–transformed leukemic progenitors after treatment with the indicated concentration of UNC1999 for 8 days. Black bar, 10 μm. (B-C) Flow cytometry analysis of c-Kit and Gr-1 after treatment with 3 μM of the indicated compounds for 8 days. (D-E) Quantification of colony-forming units from *MLL-AF9*– (D) or *MLL-ENL*–transformed leukemia progenitors (E) after serial replating into the cytokine-rich, methylcellulose medium containing DMSO or 3 μM UNC2400 or UNC1999. Data are shown as the mean ± SD of experiments in duplicate. *$P < .05$; **$P < .01$; ***$P < .001$. (F) Light micrographs show typical morphology of the single-cell colonies derived from *MLL-AF9*–transformed leukemia progenitors following serial replating in the presence of DMSO or 3 μM UNC2400 or UNC1999. Black bar, 1 mm. (G) Percentage of live and apoptotic subpopulations of *EOL-1* leukemia cells after the indicated compound treatments for 6 days. (H) Typical profiles of staining with PI and annexin V after treatment of *EOL-1* cells with DMSO or the indicated concentration of UNC1999 for 6 days. PI, propidium iodide.

(available on the *Blood* Web site). UNC1999 and derivatives were dissolved in dimethylsulfoxide (DMSO) as 5 mM stocks before use.

Mass spectrometry–based quantification

Total histones were prepared and subject to mass spectrometry analysis as previously described.[30]

Purification, culture, and leukemia transformation of primary hematopoietic stem and progenitor cells

Wild-type Balb/C mice and *p16Ink4a*$^{-/-}$;*p19Arf*$^{-/-}$ knockout mice (strain number 01XB2) were purchased from the NCI at Frederick Mouse Repository. Bone marrow isolated from femur and tibia of mice was subject to lineage-negative enrichment, followed by cytokine stimulation and retroviral transduction of oncogenes (*MLL-AF9*) as described.[31,32] Freshly immortalized leukemia progenitor cell lines were generated, characterized, and maintained with the previously described procedures.[31-33] The detailed procedures for flow cytometry, antibody and immunoblot, and various assays of cell proliferation, Wright-Giemsa staining, colony-forming units by serial replating, cell-cycle profiling, and apoptosis are described in supplemental Materials.

Microarray analysis

Total RNA was isolated followed by quantification of the transcript expression levels with Affymetrix GeneChip MOGene_2.1_ST. After RNA hybridization, scanning, and signal quantification (UNC Genomics Core), hybridization signals were retrieved, followed by normalization, differential expression analysis, gene ontology (GO) analysis, gene set enrichment analysis (GSEA), and statistical analysis using GeneSpring Analysis Platform GX12.6 (Agilent Technologies) as described.[34] GSEA was also carried out with the downloaded GSEA software (www.broadinstitute.org/gsea) by exploring the Molecular Signatures Database (www.broadinstitute.org/gsea/msigdb/annotate.jsp).

ChIP followed by deep sequencing

Chromatin samples used for chromatin immunoprecipitation (ChIP) followed by deep sequencing (ChIP-Seq) were prepared using a previously described protocol,[35] followed by antibody enrichment, library generation, and parallel sequencing using an Illumina HiSeq-2000 Sequencer (UNC High-throughput Sequencing Facility) as described before.[36] The detailed procedures of ChIP-Seq data alignment, filtration, peak calling and assignment, and cross-sample comparison and analysis are described in supplemental Materials.

Real-time PCR

The quantitative polymerase chain reaction (qPCR) following reverse transcription (RT-qPCR) or ChIP (ChIP-qPCR) was carried out as previously described.[34] Information on primers used is described in supplemental Table 6.

In vivo leukemogenic assay and compound treatment

MLL-AF9–induced murine leukemia was generated as previously described,[31,32] followed by compound treatment. The powder of UNC1999 (verified by high-performance liquid chromatography and mass spectrometry) was slowly dissolved and incorporated in vehicle (0.5% of sodium carboxymethylcellulose and 0.1% of Tween 80 in sterile water) with continuous trituration by a pestle in a mortar. UNC1999 or vehicle was administered by oral gavage twice daily at a dose of 50 mg/kg. The detailed descriptions of murine leukemia generation, compound usage and delivery, animal care and dissection, and pathological analysis are described in supplemental Materials.

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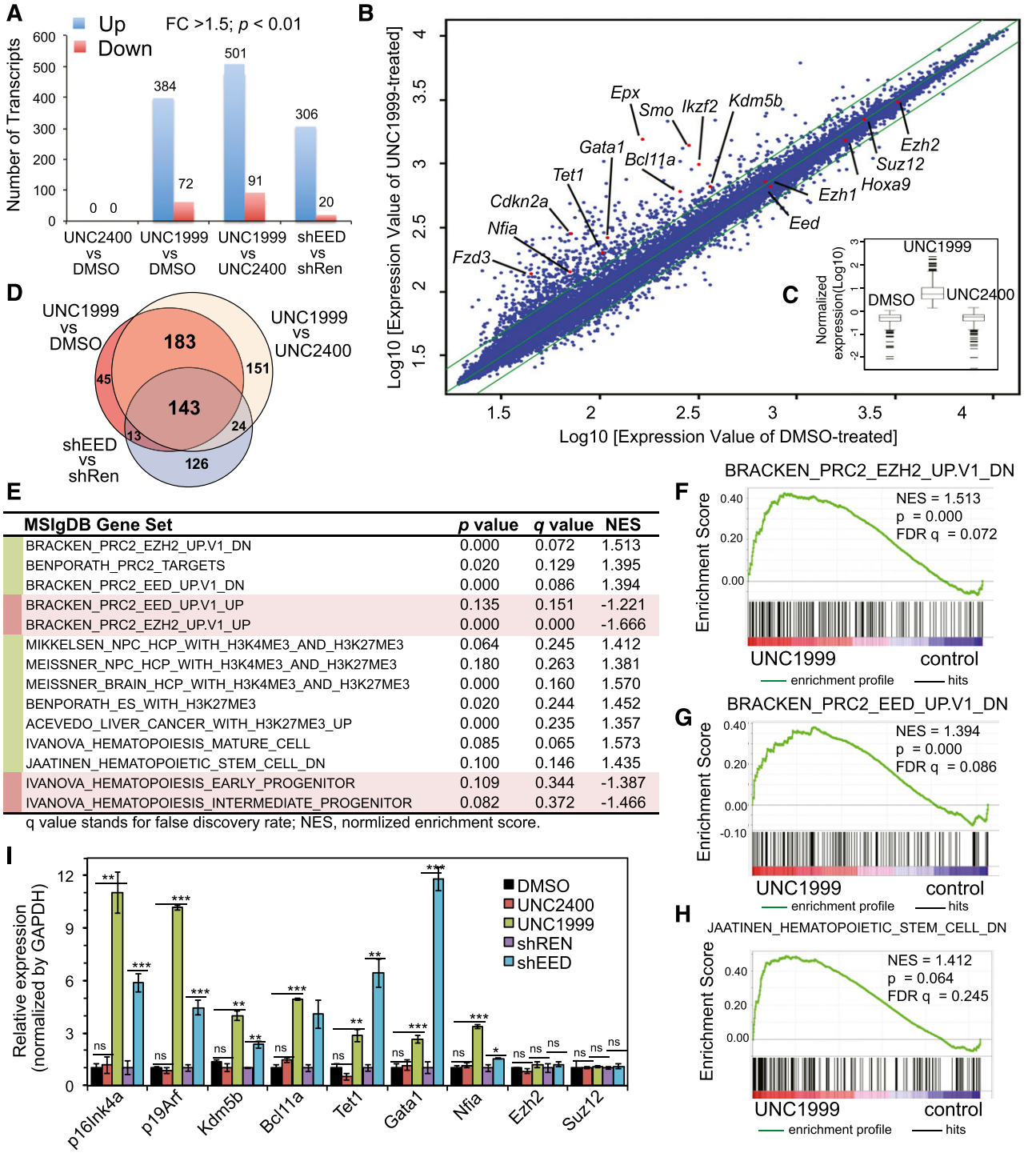

MSIgDB Gene Set	*p* value	*q* value	NES
BRACKEN_PRC2_EZH2_UP.V1_DN	0.000	0.072	1.513
BENPORATH_PRC2_TARGETS	0.020	0.129	1.395
BRACKEN_PRC2_EED_UP.V1_DN	0.000	0.086	1.394
BRACKEN_PRC2_EED_UP.V1_UP	0.135	0.151	-1.221
BRACKEN_PRC2_EZH2_UP.V1_UP	0.000	0.000	-1.666
MIKKELSEN_NPC_HCP_WITH_H3K4ME3_AND_H3K27ME3	0.064	0.245	1.412
MEISSNER_NPC_HCP_WITH_H3K4ME3_AND_H3K27ME3	0.180	0.263	1.381
MEISSNER_BRAIN_HCP_WITH_H3K4ME3_AND_H3K27ME3	0.000	0.160	1.570
BENPORATH_ES_WITH_H3K27ME3	0.020	0.244	1.452
ACEVEDO_LIVER_CANCER_WITH_H3K27ME3_UP	0.000	0.235	1.357
IVANOVA_HEMATOPOIESIS_MATURE_CELL	0.085	0.065	1.573
JAATINEN_HEMATOPOIETIC_STEM_CELL_DN	0.100	0.146	1.435
IVANOVA_HEMATOPOIESIS_EARLY_PROGENITOR	0.109	0.344	-1.387
IVANOVA_HEMATOPOIESIS_INTERMEDIATE_PROGENITOR	0.082	0.372	-1.466

q value stands for false discovery rate; NES, normlized enrichment score.

Figure 4. UNC1999, and not UNC2400, derepresses the PRC2 gene targets. (A) Summary of the upregulated (blue) and downregulated (red) transcripts in 2 independent *MLL-AF9*–transformed leukemia lines after a 5-day treatment with 3 μM of compounds or after knockdown of *EED* vs *Renilla*, as identified by microarray analysis with a cutoff of FC of >1.5 and a *P* value of <.01. (B) Scatter plot to compare the global gene expression pattern in *MLL-AF9*–transformed leukemia cells following DMSO (x-axis) vs UNC1999 treatment (y-axis). Plotted are Log10 values of the signal intensities of all transcripts on gene microarrays after normalization. The flanking lines in green indicate 1.5-fold change in gene expression. (C) Boxplots showing the expression levels of upregulated transcripts in the compound- vs DMSO-treated samples. Y-axis represents the Log10 value of signal intensities detected by microarray. (D) Venn diagram of the upregulated transcripts shown in panel A. (E) Summary of GSEA using the MSIgDB. Green and red indicate the positive and negative correlation to UNC1999-treated cells, respectively. (F-H) GSEA revealing significant enrichment of the EZH2-repressed (F) or EED-repressed gene signatures (G) and those negatively associated with hematopoietic stem cells (H) in the UNC1999- vs DMSO-treated cells. (I) RT-qPCR detects relative expression levels of the indicated genes in *MLL-AF9*–transformed leukemia cells following treatment with 3 μM of compounds or *EED* knockdown (shEED) for 5 days. Y-axis represents fold-change after normalization to *GAPDH* and to control (DMSO treatment or *Renilla* knockdown [shRen]), and error bars represent SD of triplicates. **P* < .05; ***P* < .01; ****P* < .001. FC, fold-change; FDR, false discovery rate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MSIgDB, Molecular Signatures Database; NES, normalized enrichment score; Ren, *Renilla*; shEED, shRNA against EED; shREN, shRNA against Renilla.

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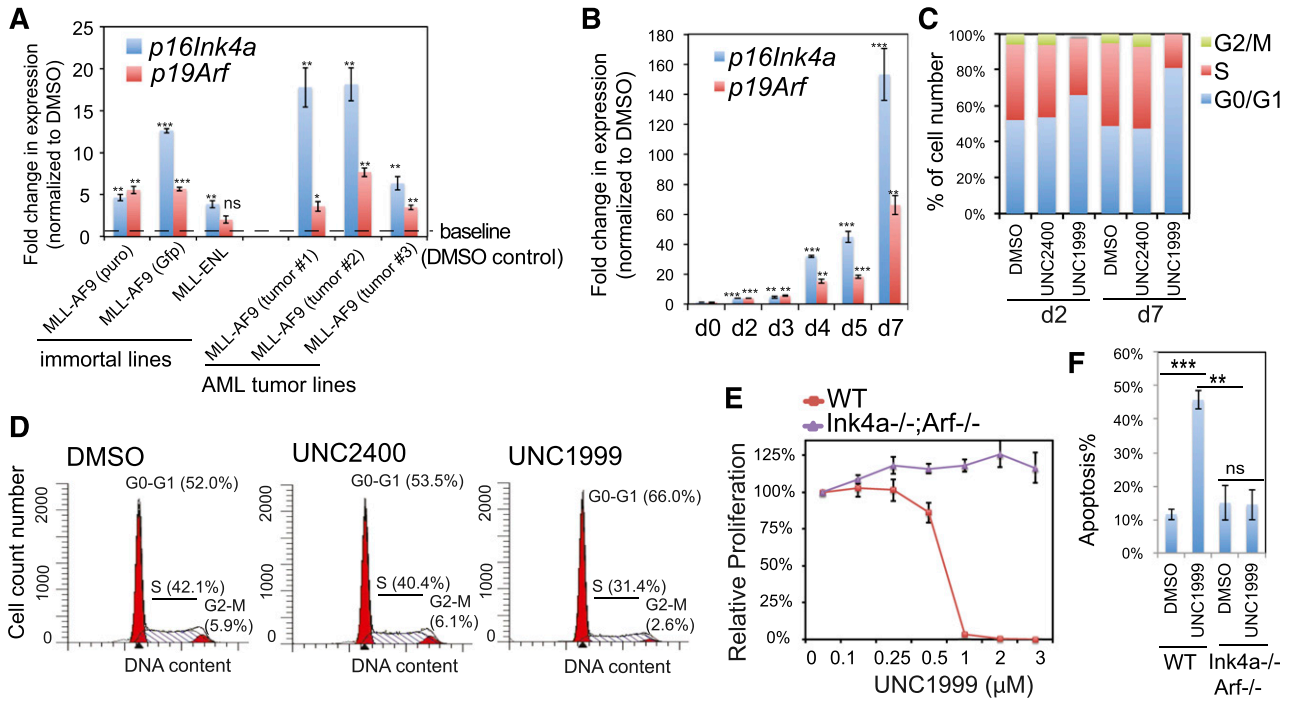

Figure 5. *Cdkn2a* reactivation plays a critical role in UNC1999-mediated growth inhibition. (A) Change in *p16Ink4a* and *p19Arf* gene expression following a 3-day treatment with 3 μM UNC1999 among 6 independent murine leukemia lines, either freshly immortalized by *MLL-AF9* or *MLL-ENL* (left) or derived from *MLL-AF9*–induced primary murine leukemia (right). Y-axis represents fold-change in gene expression after normalization to *GAPDH* and DMSO treatment, and error bars represent SD of triplicates. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$. (B) RT-qPCR shows time-dependent derepression of *p16Ink4a* and *p19Arf* by UNC1999 in a leukemia line derived from *MLL-AF9*–induced primary tumors. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$. (C) Summary of cell-cycle status of *MLL-AF9*–transformed murine leukemia progenitors following 2-day or 7-day treatment with DMSO, or 3 μM UNC2400 or UNC1999. (D) Representative histograms showing DNA contents measured by PI staining of *MLL-AF9*–transformed leukemia cells after treatment with 3 μM of compounds for 2 days. (E) Relative proliferation of *MLL-AF9*–transformed murine leukemia cells, either wild-type (red) or *p16Ink4a*$^{-/-}$/*p19Arf*$^{-/-}$ (purple), after treatment with various concentrations of UNC1999 for 12 days. Y-axis, presented as the mean of triplicates ± SD, represents the relative percentage of cell numbers after normalization to DMSO treatment. (F) Summary of apoptotic induction in *MLL-AF9*–transformed murine leukemia progenitors, either wild-type (WT) or *p16Ink4a/p19Arf*-deficient, following a 6-day treatment with 3 μM of compounds as assayed by PI staining. $^{**}P < .01$; $^{***}P < .005$. WT, wild type.

Statistical analysis

Data are presented as the mean ± standard deviation (SD) for 3 independent experiments unless otherwise noted. Statistical analysis was performed with the Student *t* test, except for nonparametric analysis that used the log-rank (Mantel-Cox) test.

Results

A small-molecule UNC1999, and not its inactive analog UNC2400, selectively and potently suppresses H3K27me3/2

Previously, using an in vitro methyltransferase assay, we have shown that UNC1999 (Figure 1A) exhibits highly selective and potent inhibition of EZH2 and EZH1 over other unrelated methyltransferases with half-maximal inhibitory concentration (IC_{50}) for EZH2 and EZH1 measured at <10 nM (Figure 1B) and 45 nM, respectively.[25] UNC2400, an inactive analog compound (with IC_{50} of >13 000 nM), was generated by modifying UNC1999 with 2 N-methyl groups (Figure 1A-B). Via docking studies with the recently solved apo structure of the EZH2 SET domain,[37,38] we found that the 2 N-methyl modifications presumably disrupt the critical hydrogen bonds formed by UNC1999 with the side-chain carbonyl of *Asn*688 and the N-terminal nitrogen of *His*689 of EZH2 (supplemental Figure 1A). Modifying UNC1999 with both N-methyl groups is required to abrogate its potency because UNC3142 and UNC1756 (supplemental Figure 1B), 2 compounds with a single N-methyl modification at either position, merely modestly interfered with EZH2-mediated methylation (Figure 1B).

To assess the effect of our active vs inactive compounds on the landscape of histone modifications, we used mass spectrometry proteomic techniques[30] to quantify histone modification levels following compound treatment of a murine leukemia line established by *MLL-ENL*,[32] a common form of *MLL* rearrangements.[26,27] Of 55 detected histone peptides carrying the single or combinatorial modification, only peptides covering the H3 residues 27-40 were found altered in relative abundance with fold-change of >2 following UNC1999 vs DMSO treatment (Figure 1C, blue; supplemental Table 1). Peptides with a single H3K27me3 or H3K27me2 modification showed the greatest decreases. H3(27-40) peptide can also be modified by H3K36 methylation and, indeed, following UNC1999 treatment, several peptides with dual methylations of H3K27 and H3K36 were either undetectable (H3K27me3-K36me2) or found decreased (H3K27me3-K36me1 and H3K27me2-K36me2/1) (Figure 1C, blue; supplemental Table 1). Due to H3K27 "demethylation" by UNC1999 on these dually methylated peptides, the relative abundance of certain peptide species bearing H3K36me1/2 (supplemental Figure 1C-D, see increases in blue in bar graph, UNC1999 vs mock) was found increased accordingly (Figure 1C; supplemental Table 1), a phenomenon also seen in cells deficient in *Suz12*, an essential subunit of PRC2 (supplemental Figure 1E, blue in bar graph).[39] Overall, global H3K36me1/2 does not show significant change as examined by mass spectrometry (supplemental Figure 1F) and immunoblot (supplemental Figure 1G); indeed, at the concentrations applied to cells (<3 μM), UNC1999 had no effect on all 4 known H3K36-specific methyltransferases (supplemental Figure 1H-K). These findings collectively demonstrated specific targeting of PRC2 by UNC1999. As a result, the overall percentage of H3K27me3 and

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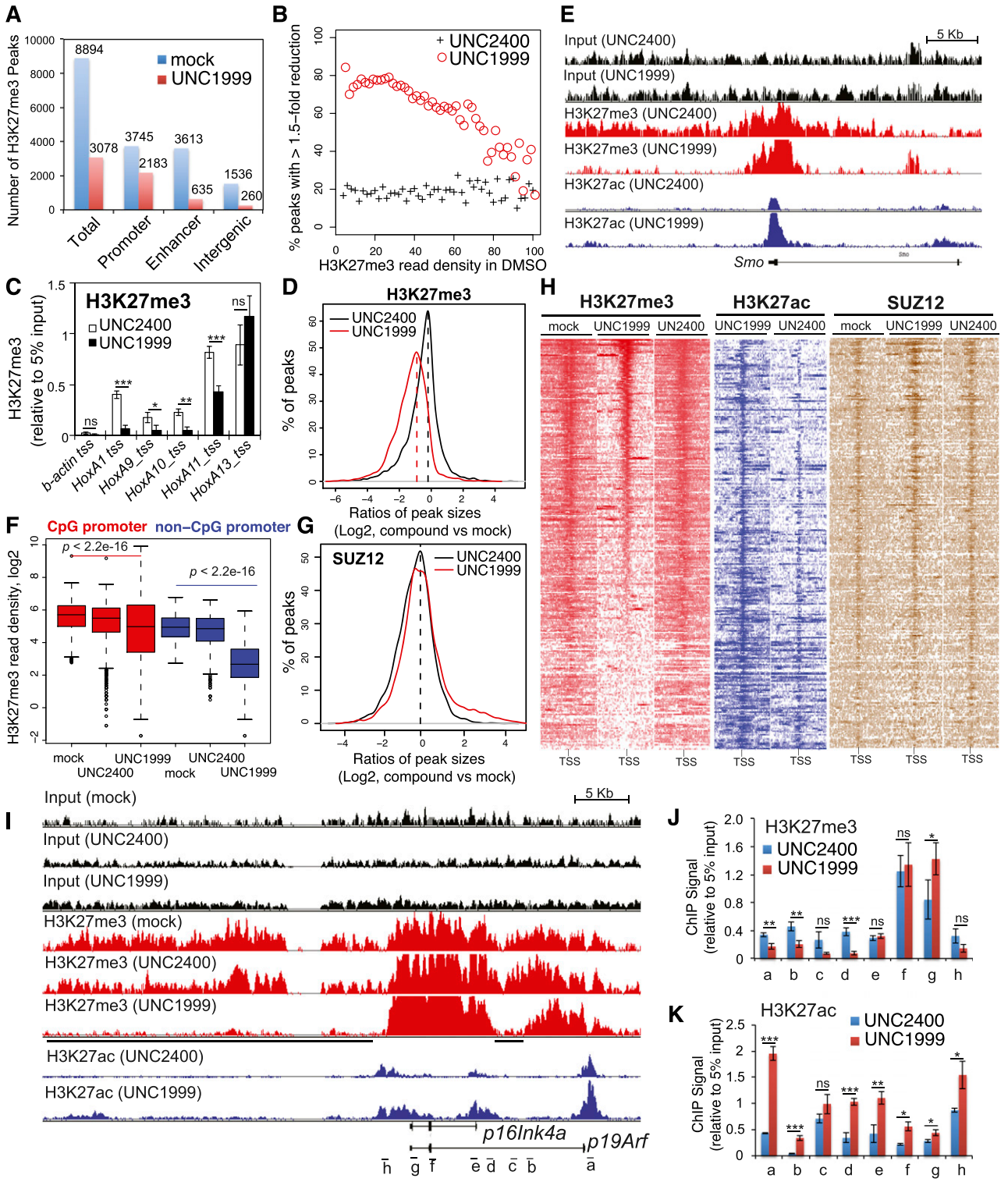

Figure 6. ChIP-Seq reveals UNC1999-induced loss of H3K27me3 and concurrent gain of H3K27ac in *MLL-AF9*–transformed leukemia progenitors. (A) Summary of H3K27me3 peaks showing loss in the UNC1999- (red) vs mock-treated (blue) samples. X-axis indicates all H3K27me3 peaks (left) or those associated with promoters, enhancers, or intergenic regions. (B) The fractions of H3K27me3 peaks showing reduction in ChIP-Seq signals by 1.5-fold or more in UNC1999-treated (red circle) or UNC2400-treated (cross) samples in comparison with mock treatment. The H3K27me3 peaks and their densities shown on x-axis were first defined and then grouped by the number of ChIP-Seq reads identified in the mock-treated sample; y-axis represents the fraction in each group of H3K27me3 peaks that show reduction by >1.5-fold in the compound- vs mock-treated samples, after normalization of ChIP-Seq reads to the sequencing depths and peak sizes. (C) ChIP-qPCR detects H3K27me3 at the TSS of several *Hox-A* genes in *MLL-AF9*–transformed leukemia progenitors after treatment with 3 μM UNC2400 or UNC1999 for 4 days. ChIP signals (y-axis) were normalized to 5% of input and presented as mean ± SD. TSS of *β-actin* was used as negative control. *$P < .05$; **$P < .01$; ***$P < .001$. (D) Plot showing a global reduction in the H3K27me3 peak sizes following UNC1999 treatment (red). X-axis shows the ratios (in their Log2 values) of peak sizes following UNC1999 (red) or UNC2400 (black) treatment in comparison with mock; y-axis shows the relative fraction of peaks at each individual ratio. The dashed vertical lines mark the mean value of peak size ratios. (E) IGB view showing the distribution of input (black), H3K27me3 (red) and H3K27ac (blue) ChIP-Seq read densities (normalized by the ChIP-seq read depths) at the Smoothened (*Smo*) gene in *MLL-AF9* leukemia progenitors after treatment with 3 μM UNC2400 or UNC1999 for 4 days. (F) Boxplots showing a significantly greater reduction of ChIP-Seq enrichment at the non-CpG- than the CpG-contained promoter associated H3K27me3 peaks after UNC1999 treatment in comparison with mock treatment. (G) Plot showing

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H3K27me2 was reduced from 8.5% and 30.9% of total H3 in mock-treated cells, respectively, to 1.3% and 7.1% in UNC1999-treated cells, H3K27me1 slightly altered from 45.6% to 30.7%, whereas the nonmethylated H3K27 increased accordingly from 14.8% to 60.4% (Figure 1D). Consistent with antagonism between PRC2 and H3K27 acetylation (H3K27ac),[40] we detected significantly increased H3K27ac after UNC1999 treatment (Figure 1C; supplemental Table 1).

None of these alterations were seen following UNC2400 treatment (Figure 1C, red; supplemental Table 1). By immunoblot (Figure 1E) and flow cytometry (Figure 1F-G; supplemental Figure 1L-N), we verified "erasure" of H3K27me3/2 and concurrent elevation of H3K27ac by UNC1999, its negligible effects on other histone methylations, and undetectable effects by UNC2400. UNC1999-mediated suppression of H3K27me3 was time- and concentration-dependent (Figure 1F-G; supplemental Figure 1L-N). UNC1999 did not alter total levels of PRC2 (supplemental Figure 1O-P) or chromatin-bound EZH2 and EZH1 (Figure 1H), and did not affect the stability or assembly of PRC2 (Figure 1I), indicating that UNC1999 acts primarily via enzymatic inhibition of EZH2 and EZH1 on chromatin.

Taken together, UNC1999 induces potent and selective suppression of H3K27me3/2, whereas UNC2400 does not, highlighting them as a pair of compounds useful to manipulate both PRC2-EZH2 and PRC2-EZH1.

An EZH2 and EZH1 dual inhibitor UNC1999, but not an EZH2-selective inhibitor GSK126, effectively inhibits growth of *MLL*-rearranged leukemia

Recent studies have shown that genetic disruption of both EZH2 and EZH1 is required to inhibit growth of *MLL*-rearranged leukemia,[19,20] which prompted us to ask whether UNC1999 provides a unique way for treating *MLL*-rearranged leukemia. First, we compared the effect of UNC1999 to GSK126, a recently disclosed EZH2-selective inhibitor (with ~150-fold selectivity of EZH2 over EZH1).[22] As expected, both GSK126 and UNC1999 efficiently inhibited the global H3K27me3 in *DB* cells, a lymphoma line bearing the $EZH2^{Y641N}$ mutation,[22,25] as measured by both immunoblot (Figure 2A, top) and quantitative flow cytometry (supplemental Figure 2A), and efficiently suppressed *DB* cell proliferation (Figure 2B). However, using 3 independent *MLL-AF9*–transformed murine leukemia lines, we found that only UNC1999, and not GSK126, efficiently inhibited their H3K27me3 (Figure 2A, bottom and 2C) and suppressed cell proliferation (Figure 2D; supplemental Figure 2B-E). *MLL-AF9*–transformed murine leukemia cells coexpress *EZH2* and *EZH1* (supplemental Figure 2F). These data indicate uniqueness of UNC1999 in treating cancers that rely on PRC2-EZH2 and PRC2-EZH1 both.

Next, we applied UNC1999 to a larger panel of leukemia cell lines. All of the 10 lines bearing *MLL* rearrangements including *MLL-AF9*, *MLL-ENL, MLL-PTD*, or *MLL-AF4* showed sensitivity to UNC1999 (Figure 2E-H; supplemental Figure 2G-I) with half-maximal effective concentration (EC_{50}) ranging from 102 nM to 1.96 μM (Figure 2F). Notably, multiple *MLL*-rearranged lines demonstrated a comparable UNC1999 sensitivity to *DB* (Figure 2E-F). UNC1999 did not induce nonspecific toxicity, for it did not affect proliferation of *LOUCY* (Figure 2I), a T-cell acute lymphoblastic leukemia line without detectable H3K27me3 due to *EZH2* genomic deletion[41] (Figure 2J). Moreover, *K562*, a *BCR-ABL*–bearing myeloid leukemia line, also did not respond to UNC1999 (Figure 2E-F). UNC1999-mediated growth suppression was time-dependent and dose-dependent (Figure 2D-H). UNC2400 had no detectable effect on cell growth (Figure 2K).

Collectively, UNC1999, an EZH2 and EZH1 dual inhibitor, efficiently suppresses proliferation of *MLL*-rearranged leukemia cells that coexpress *EZH2* and *EZH1*.

UNC1999, and not UNC2400, suppresses colony-forming abilities of *MLL*-rearranged leukemia cells and promotes their differentiation and apoptosis

Wright-Giemsa staining revealed dose-dependent alterations by UNC1999 in cell morphology from leukemic myeloblasts to differentiated cells (Figure 3A; supplemental Figure 3A), which was concurrent with the increased differentiation markers and decreased c-Kit, a hematopoietic stem/progenitor marker (Figure 3B-C; supplemental Figure 3B). We used serial replating assays to assess the repopulating ability of clonogenic cells, an ex vivo indicator of leukemia stem cells.[42] Following treatment with UNC1999, and not DMSO or UNC2400, *MLL-AF9*– or *MLL-ENL*–transformed leukemia cells exhibited a dramatic reduction in the number of outgrowing colonies (Figure 3D-E) and morphologic alterations from the primarily large and compact colonies to the small and diffuse ones characteristic of differentiated cell clusters (Figure 3F). We also examined cell viability and found time- and concentration-dependent induction of apoptosis in several tested lines after treatment with UNC1999, and not UNC2400 (Figure 3G-H; supplemental Figure 3C). Taken together, UNC1999, but not UNC2400, suppresses growth of *MLL*-rearranged leukemia by inhibiting repopulating ability and promoting cell differentiation and apoptosis.

Identification of UNC1999-responsive gene signatures in *MLL*-rearranged leukemia

To dissect the underlying mechanisms for the UNC1999-induced anti-leukemia effect, we performed microarray analysis with 2 independent *MLL-AF9*–transformed murine leukemia lines following compound treatments. UNC1999 altered the expression of a few hundred transcripts (Figure 4A-B; supplemental Figure 4A, supplemental Table 2), and consistent with the silencing role of PRC2,[43] significantly more genes showed upregulation than downregulation after UNC1999 treatment (Figure 4A-B; supplemental Figure 4A). In contrast, UNC2400 induced little changes (Figure 4A,C;

Figure 6 (continued) the relative size of SUZ12 peaks after compound treatments. X-axis shows the ratios (in their Log2 values) of peak sizes following UNC1999 (red) or UNC2400 (black) treatment in comparison with mock; y-axis shows the relative fraction of peaks at each individual ratio. The dashed vertical lines mark the mean value of peak size ratios. (H) Heatmap showing the ChIP-Seq read densities of H3K27me3 (red), H3K27ac (blue), and SUZ12 (brown) across the TSS (±20 kb) of upregulated genes following UNC1999 vs mock treatment (Figure 4A). Color represents the degree of ChIP-Seq signal enrichment, with the lowest set to white. The data indicate that a large majority (top of the heatmaps) of the UNC1999-derepressed genes contains H3K27me3 across TSS prior to compound treatment, and following UNC1999 treatment, H3K27me3 peaks become narrower and sharper. (I) IGB profiles showing the distribution of ChIP-Seq read densities (normalized by the ChIP-seq read depths) for input (black), H3K27me3 (red), and H3K27ac (blue) at *p16Ink4a* and *p19Arf*. Black bars under the track of H3K27me3 (UNC1999) mark the regulatory regions showing loss or reduction of H3K27me3 after UNC1999 treatment in comparison with mock or UNC2400. (J-K) ChIP-qPCR of H3K27me3 (J) and H3K27ac (K) across the *Cdkn2a* locus in *MLL-AF9*–transformed leukemia progenitors after treatment with 3 μM UNC2400 (blue) or UNC1999 (red) for 4 days. The genomic organization of *p16Ink4a* and *p19Arf* and positions of each ChIP PCR amplicon (labeled alphabetically, not drawn to scale) are depicted at the bottom of panel I. ChIP signals (y-axis) from independent experiments were normalized to input and presented as mean ± SD. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$. IGB, Integrated Genome Browser.

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supplemental Table 2), demonstrating its overall inactivity in transcriptional modulation. Importantly, the transcripts upregulated by UNC1999 largely overlapped those after knockdown of *EED*, a common cofactor of EZH2 and EZH1, demonstrating on-target effects of UNC1999 (Figure 4D; supplemental Figure 4A-C, supplemental Table 2-3). GSEA revealed significant enrichment of PRC2-repressed genes (Figure 4E-G) and those associated with H3K27me3 (supplemental Figure 4D-E) or myeloid differentiation (Figure 4E,H) in UNC1999- vs DMSO-treated cells. GO analysis showed UNC1999-derepressed genes enriched with pathways related to development, myeloid differentiation, and proliferation (supplemental Figure 4F), which are the hallmark of polycomb targets.[43] For example, similar to *EED* knockdown (supplemental Table 3), UNC1999 treatment derepressed the differentiation-associated (*Epx*), proliferation-associated (*Cdkn2a*), and development-associated genes (*Bcl11a*, *Ikzf2*, *Gata1*, *Tet1*, *Kdm5b*, *Smo*, *Fzd3*), whereas expression of all PRC2-encoding genes were unaltered (Figure 4B). By RT-qPCR, we verified the gene expression changes following UNC1999 vs UNC2400 treatments or after *EED* knockdown (Figure 4I).

Taken together, treatment of *MLL*-rearranged leukemias with UNC1999, an EZH2 and EZH1 dual inhibitor, derepresses their PRC2 gene targets.

Cdkn2a reactivation is crucial for UNC1999-induced growth suppression

We performed similar gene array analysis either with milder compound treatment or with *MLL-ENL*–transformed leukemia cells and identified a common UNC1999-responsive signature, which included *Cdkn2a* (supplemental Table 4). We closely examined *Cdkn2a* because this polycomb target encodes 2 crucial cell-cycle regulators, *p16Ink4a* and *p19Arf*. UNC1999 consistently induced reactivation of *p16Ink4a* and *p19Arf* in multiple lines bearing *MLL-AF9* or *MLL-ENL,* and such derepression was concentration- and time-dependent (Figure 5A-B; supplemental Figure 5A). *Cdkn2a* reactivation was modest after 2-day treatment and dramatic 7 days posttreatment, with >150-fold and >60-fold upregulation of *p16Ink4a* and *p19Arf* observed in sensitive lines, respectively (Figure 5A-B). UNC1999 induced cell-cycle arrest at the G1-to-S transition (Figure 5C-D; supplemental Figure 5B). In contrast, UNC2400 did not alter the expression of *Cdkn2a* (Figure 4C,I) or cell-cycle progression (Figure 5C-D; supplemental Figure 5B). To examine whether UNC1999-induced phenotypes depend on *Cdkn2a*, we derived several *MLL-AF9*–transformed leukemia lines with bone marrow from mice deficient in *p16Ink4a* and *p19Arf* (supplemental Figure 5C). Compared with their wild-type counterparts, these $p16Ink4a^{-/-}/p19Arf^{-/-}$ leukemia cells no longer responded to UNC1999 with no detectable change in their proliferation (Figure 5E) or apoptosis (Figure 5F) following treatment. Collectively, *Cdkn2a* is a critical downstream mediator of UNC1999-induced growth inhibition.

ChIP-Seq reveals UNC1999-induced local suppression of H3K27me3 and concurrent gain of H3K27ac in *MLL*-rearranged leukemia

To further dissect how UNC1999 alters the chromatin landscape of *MLL-AF9*–transformed leukemia progenitors, we used ChIP-Seq to profile distribution of H3K27me3 and its antagonizing H3K27ac. We found a global reduction of H3K27me3 following UNC1999 treatment: ~66% of a total of 8894 H3K27me3 peaks showed complete loss (Figure 6A) whereas 8% showed increases (>1.5-fold, data not shown). ChIP-Seq also unveiled preferential removal of H3K27me3 by UNC1999 at loci containing a relatively lower level of H3K27me3 whereas those harboring the highest H3K27me3 remained generally intact (Figure 6B). For example, UNC1999 completely "erased" a domain with lower H3K27me3 upstream of *HoxA1* whereas a domain with higher H3K27me3 covering *HoxA11-A13* was unaltered (supplemental Figure 6A red); using ChIP followed by qPCR, we verified such site-specific "demethylation" by UNC1999 at *Hox-A* genes (Figure 6C). Furthermore, H3K27me3 peaks associated with distal nonpromoter regulatory elements, including enhancers and intergenic regions, often demonstrate complete loss, whereas H3K27me3 peaks associated with proximal promoters are largely retained (Figure 6A; supplemental Figure 6D), despite a shrinking in their average peak size (Figure 6D) or signal reduction at peak shoulders, as exemplified by developmental genes *Smo* (Figure 6E, red), *Evx1*, *Bcl11a*, and *Fzd3* (supplemental Figure 6A-C, red). In addition, we found UNC1999 preferentially affected non-CpG promoters compared with CpG promoters (Figure 6F). We also performed ChIP-Seq of SUZ12, an essential cofactor of EZH2 and EZH1, and did not observe significant decreases in SUZ12 binding following compound treatments (Figure 6G; supplemental Figure 6E), which is consistent with our finding that UNC1999 does not affect PRC2 complex stability (Figure 1I). To correlate the transcriptome alteration with ChIP-Seq, we examined UNC1999-derepressed genes. Indeed, following UNC1999 vs mock or UNC2400 treatment, these genes displayed either loss or shrinking of H3K27me3 centered on transcriptional start sites (TSSs) (Figure 6H, red) and concurrent gain in H3K27ac (Figure 6H, blue), whereas the associated SUZ12 was not decreased (Figure 6H, brown). We then closely examined *Cdkn2a*, a crucial UNC1999-responsive gene. Again, ChIP-Seq revealed preferential "erasure" of H3K27me3 by UNC1999 at multiple regulatory elements (Figure 6I, black lines under the track of "H3K27me3-UNC1999") of *p16Ink4a* or *p19Arf* without altering *Cdkn2a*-associated SUZ12 (supplemental Figure 6F); TSS-associated H3K27ac was significantly increased (Figure 6I, blue). By ChIP-qPCR, we verified site-specific decreases in H3K27me3 (Figure 6J) and increases in H3K27ac (Figure 6K) across *Cdkn2a* after UNC1999 vs UNC2400 treatment.

Collectively, UNC1999, but not UNC2400, preferentially "erases" H3K27me3 associated with distal regulatory regions such as enhancers and reshapes the landscape of H3K27me3 vs H3K27ac at proximal promoters in *MLL-AF9*–transformed leukemia cells, leading to gene derepression.

UNC1999 prolongs survival of an *MLL-AF9*–induced murine leukemia model in vivo

We generated murine leukemia with *MLL-AF9* followed by bone marrow transplantation of primary tumors to syngeneic recipients, which developed aggressive leukemia with a consistent latency of 20 to 35 days (Figure 7A, blue). To examine the effect of UNC1999 on in vivo leukemogenesis, we administered either vehicle or 50 mg/kg UNC1999 by oral gavage to mice twice per day and starting from 7 days posttransplantation when the white blood cell (WBC) counts in peripheral blood started to accumulate when compared with nonengrafted controls. We then monitored leukemia progression by periodic assessment of peripheral blood samples and found that, despite steady accumulation of WBCs in both vehicle- and UNC1999-treated cohorts, UNC1999-treated animals displayed significant reduction in WBC counts (Figure 7B-D), indicating a delayed leukemic progression. Indeed, UNC1999-treated leukemic mice exhibited a significantly prolonged survival, with a latency of 36.6 ± 11.7 days in contrast to 24 ± 6.7 days for vehicle-treated

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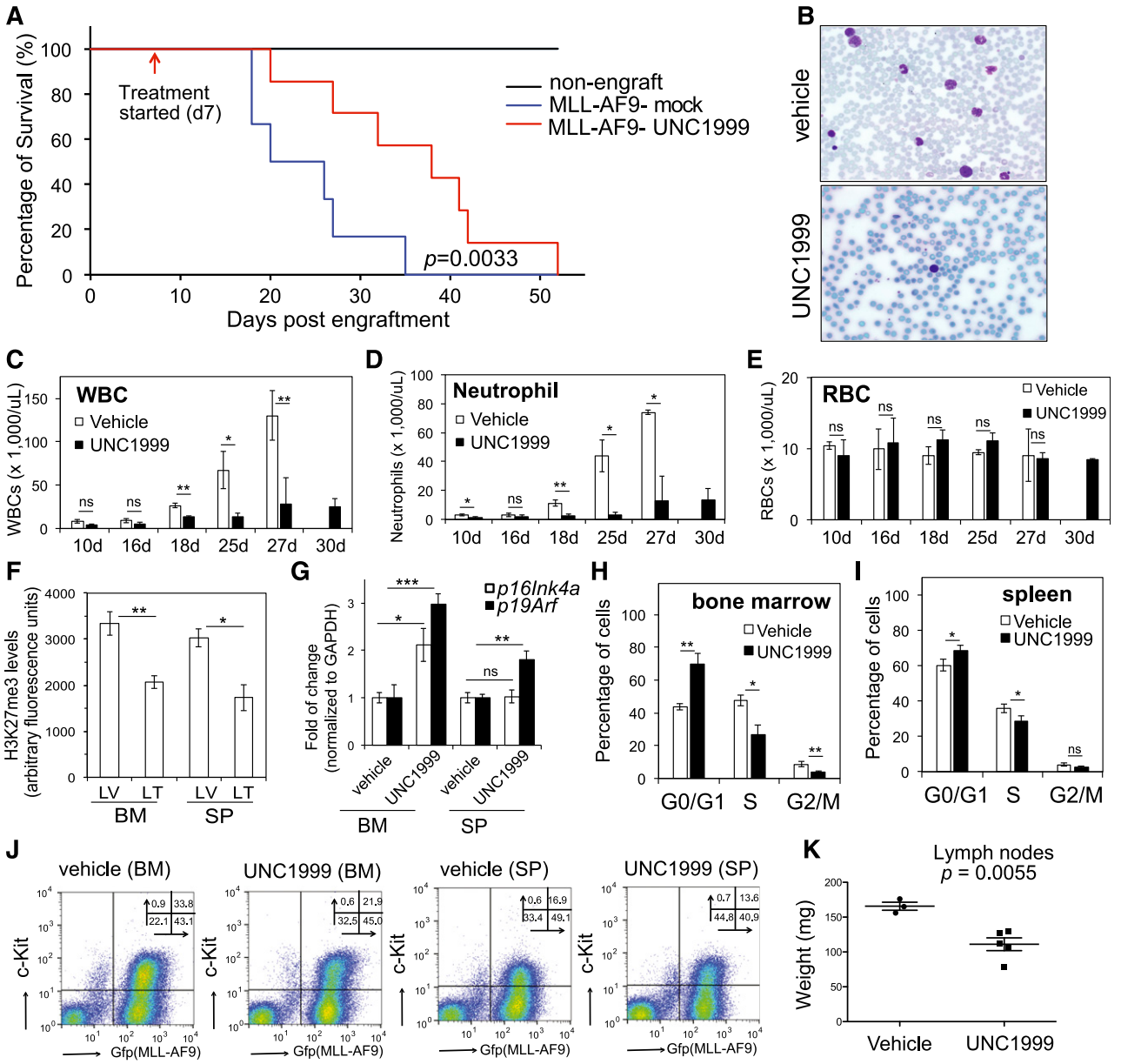

Figure 7. UNC1999 prolongs survival of *MLL-AF9*–induced murine leukemia models in vivo. (A) Kaplan-Meier curve showing leukemia kinetics after transplantation of *MLL-AF9*–induced primary murine leukemia into syngeneic mice. Starting from day 7 posttransplantation, mice received oral administration of either vehicle (blue) or 50 mg/kg UNC1999 (red) twice per day. Black lines (top) represent nontransplanted normal mice treated with vehicle or UNC1999. Cohort size, 6 to 7 mice. (B) Typical Wright-Giemsa staining images of the peripheral blood smears prepared from the vehicle- (top) and UNC1999-treated (bottom) leukemia mice 25 days posttransplantation. (C-E) Summary of counts of the WBCs (C), neutrophils (D), and RBCs (E) in the peripheral blood of vehicle- (white) or UNC1999-treated (black) leukemia mice at the indicated date posttransplantation. $*P < .05$; $**P < .01$; $***P < .001$. (F) Summary of H3K27me3 levels in cells isolated from bone marrow or spleen of vehicle- (LV) and UNC1999-treated (LT) leukemia mice as quantified by flow cytometry. $*P < .05$; $**P < .01$. (G) Fold-change in *p16Ink4a* and *p19Arf* gene expression in cells isolated from bone marrow or spleen of the UNC1999-treated (black) leukemia mice in comparison with vehicle-treated (white). $*P < .05$; $**P < .01$. (H-I) Summary of cell-cycle status of cells isolated from bone marrow (H) or spleen (I) of vehicle- (white) and UNC1999-treated (black) leukemia mice 25 days posttransplantation. $*P < .05$; $**P < .01$. (J) Flow cytometry (performed 25 days posttransplantation) detects leukemia cells (labeled by bicistronic *GFP* expression in x-axis) and their *c-Kit* expression (y-axis) in the bone marrow or spleen after treating mice with either vehicle or 50 mg/kg UNC1999. (K) Comparison of sizes of lymph nodes isolated from the *MLL-AF9*–induced leukemia mice after treatment with either vehicle or 50 mg/kg UNC1999. BM, bone marrow; GFP, green fluorescent protein; LT, UNC1999-treated leukemia mice; LV, vehicle-treated leukemia mice; RBC, red blood cell; SP, spleen.

mice (Figure 7A, $P = .0033$). UNC1999 treatment did not affect the counts of red blood cells (Figure 7E) or platelets (supplemental Figure 7A). We closely examined leukemia cells collected from moribund mice. Compared with vehicle, UNC1999 treatment significantly decreased H3K27me3 in cells isolated from bone marrow and spleen (Figure 7F), significantly elevated their *p16Ink4a* or *p19Arf* expression (Figure 7G), and caused the G1-to-S cell-cycle arrest (Figure 7H-I; supplemental Figure 7B). UNC1999 treatment also significantly reduced the total number of leukemia cells (labeled by bicistronic *GFP* expression) or c-Kit–positive leukemia progenitors (c-Kit$^+$/GFP$^+$) in bone marrow and spleen (Figure 7J; supplemental Figure 7C-D). Furthermore, the size of lymph nodes was significantly smaller in UNC1999- vs vehicle-treated mice (Figure 7K); both cohorts showed similar severity of splenomegaly.

Collectively, oral administration of UNC1999 delays *MLL-AF9*–induced leukemogenicity in vivo and our EZH2 and EZH1 dual inhibitor provides a new therapeutics for *MLL*-rearranged leukemia.

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Discussion

Our study represents the first to show specific enzymatic inhibition of EZH2 and EZH1 by oral delivery of a small-molecule compound as a promising therapeutic intervention for *MLL*-rearranged leukemia, a genetically defined malignancy with poor prognosis. Previously, several methods were reported to disrupt PRC2 function in cancer. 3-Deazaneplanocin A (DZNep), an inhibitor of S-adenosylhomocysteine hydrolase that depletes PRC2 via an unclear mechanism,[44] demonstrated a tumor-suppressive effect in cancers including *MLL*-rearranged leukemia[45]; however, increasing evidence has indicated that DZNep lacks specificity.[46,47] Inhibitors highly selective to EZH2[21-23] or to EZH2 and EZH1[25,48] have been discovered, some of which demonstrated early success in treating *EZH2*-mutated lymphoma[22] and *SNF5*-inactivated malignant rhabdoid tumors.[49] Furthermore, a hydrocarbon-stapled peptide (SAH-EZH2) has recently been developed to disrupt interaction of EED with EZH2 and EZH1, leading to degradation of PRC2.[50] Prior to our study, an orally bioavailable inhibitor of PRC2 remains to be established in both in vitro and in vivo settings to treat cancer that relies on PRC2-EZH2 and PRC2-EZH1 both. Here, we used a series of proteomics, genomics, and leukemogenic approaches to show that UNC1999, an EZH2 and EZH1 dual inhibitor, suppresses growth of *MLL*-rearranged leukemia ex vivo and in vivo, whereas an EZH2-selective inhibitor GSK126 failed to efficiently inhibit H3K27me3 or proliferation of *MLL*-rearranged leukemia cells. Our translational tool may represent novel therapeutics for cancer types that coexpress *EZH2* and *EZH1*.

UNC1999-responsive genes largely overlapped the defined PRC2 targets. Derepression of PRC2 target genes associates with suppression of H3K27me3 and concurrent gain in H3K27ac. Unlike DZNep[44] or SAH-EZH2,[50] UNC1999 does not degrade PRC2 and, thus, the UNC1999-derepressed genes are likely to be silenced by PRC2 via its methyltransferase activity per se, and not via silencing factors recruited by PRC2. This difference may partly explain why a subset of genes were upregulated by *EED* knockdown and not by UNC1999 (Figure 4D). We also show that deletion of *Cdkn2a* largely reversed the UNC1999-induced antiproliferation effect in vitro, suggesting that the status of *CDKN2A* might be a useful predictor for drug resistance vs efficacy of PRC2 inhibitors. It would be also interesting to investigate into the status of *CDKN2A* among certain non-*MLL*-rearranged myeloid malignancies and T-cell acute lymphoblastic leukemia where PRC2 inactivation occurs due to the damaging mutation of *EZH2*, *EED*, or *SUZ12*.[51,52] Furthermore, we demonstrated overall inactivity of an analog compound UNC2400 in modulation of H3K27me3 or gene expression, making it an ideal chemical control for UNC1999. Thus, this study provides the first detailed molecular characterization of a pair of active vs inactive small-molecule compounds suitable for studying EZH2 and EZH1 in the in vitro and in vivo settings.

Mechanistically, UNC1999 preferentially "erases" H3K27me3 peaks associated with distal regulatory elements such as enhancers and remodels the landscape of H3K27me3 vs H3K27ac at proximal promoters and TSSs. We speculate that the local concentration and/or composition of PRC2 and associated factors may influence the efficacy of UNC1999, as we observed a higher level of SUZ12 at TSS where H3K27me3 tends to be retained following UNC1999 treatment (Figure 6H). However, the causal relationship remains to be defined. Furthermore, we noticed that UNC1999 had a weaker effect in vivo and only modestly delayed *MLL-AF9*–induced leukemogenesis. Partial in vivo efficacy has previously been seen for specific inhibitors of BRD4[53,54] and DOT1L.[55] Further optimization of potency, selectivity, and drug metabolism and pharmacokinetics are needed to enhance their in vivo antitumor effect. With accumulating evidence demonstrating a general "druggability" of many cancer-associated epigenetic "writers," "erasers," and "readers,"[1] development of epigenetic modulators shall provide novel therapeutic interventions in the near future.

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Authorship

Contribution: G.G.W. and J.J. designed the research; B.X., D.M.O., A.M., T.P., K.D.K., S.G.P., D.F.A., L.C., S.L., D.F.A., F.L., S.V.F., M.V., B.A.G., J.J., and G.G.W. performed the research; S.R., Y.L., G.G.W., and D.Z. analyzed ChIP-Seq data; B.X., D.M.O., and G.G.W. performed overall analysis and data interpretation; G.G.W. wrote the paper; and all authors checked the final version of the manuscript.

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References

1. Chi P, Allis CD, Wang GG. Covalent histone modifications—miswritten, misinterpreted and mis-erased in human cancers. *Nat Rev Cancer.* 2010;10(7):457-469.
2. Strahl BD, Allis CD. The language of covalent histone modifications. *Nature.* 2000;403(6765):41-45.
3. Greer EL, Shi Y. Histone methylation: a dynamic mark in health, disease and inheritance. *Nat Rev Genet.* 2012;13(5):343-357.
4. Esteller M. Cancer epigenomics: DNA methylomes and histone-modification maps. *Nat Rev Genet.* 2007;8(4):286-298.
5. Karberg S. Switching on epigenetic therapy. *Cell.* 2009;139(6):1029-1031.
6. Dawson MA, Kouzarides T. Cancer epigenetics: from mechanism to therapy. *Cell.* 2012;150(1):12-27.
7. Arrowsmith CH, Bountra C, Fish PV, Lee K, Schapira M. Epigenetic protein families: a new frontier for drug discovery. *Nat Rev Drug Discov.* 2012;11(5):384-400.
8. Helin K, Dhanak D. Chromatin proteins and modifications as drug targets. *Nature.* 2013; 502(7472):480-488.
9. Margueron R, Li G, Sarma K, et al. Ezh1 and Ezh2 maintain repressive chromatin through different mechanisms. *Mol Cell.* 2008;32(4): 503-518.
10. Mochizuki-Kashio M, Mishima Y, Miyagi S, et al. Dependency on the polycomb gene Ezh2 distinguishes fetal from adult hematopoietic stem cells. *Blood.* 2011;118(25):6553-6561.
11. Hidalgo I, Herrera-Merchan A, Ligos JM, et al. Ezh1 is required for hematopoietic stem cell maintenance and prevents senescence-like cell cycle arrest. *Cell Stem Cell.* 2012;11(5):649-662.
12. Caganova M, Carrisi C, Varano G, et al. Germinal center dysregulation by histone methyltransferase EZH2 promotes lymphomagenesis. *J Clin Invest.* 2013;123(12):5009-5022.
13. Béguelin W, Popovic R, Teater M, et al. EZH2 is required for germinal center formation and somatic EZH2 mutations promote lymphoid transformation. *Cancer Cell.* 2013;23(5):677-692.
14. Morin RD, Johnson NA, Severson TM, et al. Somatic mutations altering EZH2 (Tyr641) in follicular and diffuse large B-cell lymphomas of germinal-center origin. *Nat Genet.* 2010;42(2): 181-185.
15. Sneeringer CJ, Scott MP, Kuntz KW, et al. Coordinated activities of wild-type plus mutant EZH2 drive tumor-associated hypertrimethylation of lysine 27 on histone H3 (H3K27) in human B-cell lymphomas. *Proc Natl Acad Sci USA.* 2010; 107(49):20980-20985.
16. Herrera-Merchan A, Arranz L, Ligos JM, de Molina A, Dominguez O, Gonzalez S. Ectopic expression of the histone methyltransferase Ezh2 in haematopoietic stem cells causes myeloproliferative disease. *Nat Commun.* 2012;3:623.
17. Berg T, Thoene S, Yap D, et al. A transgenic mouse model demonstrating the oncogenic role of mutations in the polycomb-group gene EZH2 in lymphomagenesis. *Blood.* 2014;123(25): 3914-3924.
18. Shen X, Liu Y, Hsu Y-J, et al. EZH1 mediates methylation on histone H3 lysine 27 and complements EZH2 in maintaining stem cell identity and executing pluripotency. *Mol Cell.* 2008;32(4):491-502.
19. Neff T, Sinha AU, Kluk MJ, et al. Polycomb repressive complex 2 is required for MLL-AF9 leukemia. *Proc Natl Acad Sci USA.* 2012;109(13): 5028-5033.
20. Shi J, Wang E, Zuber J, et al. The Polycomb complex PRC2 supports aberrant self-renewal in a mouse model of MLL-AF9;Nras(G12D) acute myeloid leukemia. *Oncogene.* 2013;32(7): 930-938.
21. Knutson SK, Wigle TJ, Warholic NM, et al. A selective inhibitor of EZH2 blocks H3K27 methylation and kills mutant lymphoma cells. *Nat Chem Biol.* 2012;8(11):890-896.
22. McCabe MT, Ott HM, Ganji G, et al. EZH2 inhibition as a therapeutic strategy for lymphoma with EZH2-activating mutations. *Nature.* 2012; 492(7427):108-112.
23. Qi W, Chan H, Teng L, et al. Selective inhibition of Ezh2 by a small molecule inhibitor blocks tumor cells proliferation. *Proc Natl Acad Sci USA.* 2012; 109(52):21360-21365.
24. Knutson SK, Kawano S, Minoshima Y, et al. Selective inhibition of EZH2 by EPZ-6438 leads to potent antitumor activity in EZH2-mutant non-Hodgkin lymphoma. *Mol Cancer Ther.* 2014; 13(4):842-854.
25. Konze KD, Ma A, Li F, et al. An orally bioavailable chemical probe of the lysine methyltransferases EZH2 and EZH1. *ACS Chem Biol.* 2013;8(6): 1324-1334.
26. Dou Y, Hess JL. Mechanisms of transcriptional regulation by MLL and its disruption in acute leukemia. *Int J Hematol.* 2008;87(1):10-18.
27. Krivtsov AV, Armstrong SA. MLL translocations, histone modifications and leukaemia stem-cell development. *Nat Rev Cancer.* 2007;7(11): 823-833.
28. Slany RK. The molecular biology of mixed lineage leukemia. *Haematologica.* 2009;94(7):984-993.
29. Tanaka S, Miyagi S, Sashida G, et al. Ezh2 augments leukemogenicity by reinforcing differentiation blockage in acute myeloid leukemia. *Blood.* 2012;120(5):1107-1117.
30. Lin S, Garcia BA. Examining histone posttranslational modification patterns by high-resolution mass spectrometry. *Methods Enzymol.* 2012;512:3-28.
31. Wang GG, Song J, Wang Z, et al. Haematopoietic malignancies caused by dysregulation of a chromatin-binding PHD finger. *Nature.* 2009; 459(7248):847-851.
32. Wang GG, Cai L, Pasillas MP, Kamps MP. NUP98-NSD1 links H3K36 methylation to Hox-A gene activation and leukaemogenesis. *Nat Cell Biol.* 2007;9(7):804-812.
33. Wang GG, Calvo KR, Pasillas MP, Sykes DB, Häcker H, Kamps MP. Quantitative production of macrophages or neutrophils ex vivo using conditional Hoxb8. *Nat Methods.* 2006;3(4):287-293.
34. Cai L, Rothbart SB, Lu R, et al. An H3K36 methylation-engaging Tudor motif of polycomb-like proteins mediates PRC2 complex targeting. *Mol Cell.* 2013;49(3):571-582.
35. Lee TI, Johnstone SE, Young RA. Chromatin immunoprecipitation and microarray-based analysis of protein location. *Nat Protoc.* 2006;1(2):729-748.
36. Goldberg AD, Banaszynski LA, Noh KM, et al. Distinct factors control histone variant H3.3 localization at specific genomic regions. *Cell.* 2010;140(5):678-691.
37. Antonysamy S, Condon B, Druzina Z, et al. Structural context of disease-associated mutations and putative mechanism of autoinhibition revealed by x-ray crystallographic analysis of the EZH2-SET domain. *PLoS ONE.* 2013;8(12):e84147.
38. Wu H, Zeng H, Dong A, et al. Structure of the catalytic domain of EZH2 reveals conformational plasticity in cofactor and substrate binding sites and explains oncogenic mutations. *PLoS ONE.* 2013;8(12):e83737.
39. Jung HR, Pasini D, Helin K, Jensen ON. Quantitative mass spectrometry of histones H3.2 and H3.3 in Suz12-deficient mouse embryonic stem cells reveals distinct, dynamic post-translational modifications at Lys-27 and Lys-36. *Mol Cell Proteomics.* 2010;9(5):838-850.
40. Pasini D, Malatesta M, Jung HR, et al. Characterization of an antagonistic switch between histone H3 lysine 27 methylation and acetylation in the transcriptional regulation of Polycomb group target genes. *Nucleic Acids Res.* 2010;38(15):4958-4969.
41. Nagel S, Venturini L, Marquez VE, et al. Polycomb repressor complex 2 regulates HOXA9 and HOXA10, activating ID2 in NK/T-cell lines. *Mol Cancer.* 2010;9:151.
42. Somervaille TC, Cleary ML. Identification and characterization of leukemia stem cells in murine MLL-AF9 acute myeloid leukemia. *Cancer Cell.* 2006;10(4):257-268.
43. Margueron R, Reinberg D. The Polycomb complex PRC2 and its mark in life. *Nature.* 2011; 469(7330):343-349.
44. Tan J, Yang X, Zhuang L, et al. Pharmacologic disruption of Polycomb-repressive complex 2-mediated gene repression selectively induces apoptosis in cancer cells. *Genes Dev.* 2007;21(9): 1050-1063.
45. Ueda K, Yoshimi A, Kagoya Y, et al. Inhibition of histone methyltransferase EZH2 depletes leukemia stem cell of mixed lineage leukemia fusion leukemia through upregulation of p16. *Cancer Sci.* 2014;105(5):512-519.
46. Miranda TB, Cortez CC, Yoo CB, et al. DZNep is a global histone methylation inhibitor that reactivates developmental genes not silenced by DNA methylation. *Mol Cancer Ther.* 2009;8(6): 1579-1588.
47. Lee JK, Kim KC. DZNep, inhibitor of S-adenosylhomocysteine hydrolase, down-regulates expression of SETDB1 H3K9me3 HMTase in human lung cancer cells. *Biochem Biophys Res Commun.* 2013;438(4):647-652.
48. Garapaty-Rao S, Nasveschuk C, Gagnon A, et al. Identification of EZH2 and EZH1 small molecule inhibitors with selective impact on diffuse large B cell lymphoma cell growth. *Chem Biol.* 2013; 20(11):1329-1339.
49. Knutson SK, Warholic NM, Wigle TJ, et al. Durable tumor regression in genetically altered malignant rhabdoid tumors by inhibition of methyltransferase EZH2. *Proc Natl Acad Sci USA.* 2013;110(19):7922-7927.
50. Kim W, Bird GH, Neff T, et al. Targeted disruption of the EZH2-EED complex inhibits EZH2-dependent cancer. *Nat Chem Biol.* 2013;9(10): 643-650.
51. Simon C, Chagraoui J, Krosl J, et al. A key role for EZH2 and associated genes in mouse and human adult T-cell acute leukemia. *Genes Dev.* 2012; 26(7):651-656.
52. Shih AH, Abdel-Wahab O, Patel JP, Levine RL. The role of mutations in epigenetic regulators in myeloid malignancies. *Nat Rev Cancer.* 2012; 12(9):599-612.
53. Zuber J, Shi J, Wang E, et al. RNAi screen identifies Brd4 as a therapeutic target in acute myeloid leukaemia. *Nature.* 2011;478(7370): 524-528.
54. Filippakopoulos P, Qi J, Picaud S, et al. Selective inhibition of BET bromodomains. *Nature.* 2010; 468(7327):1067-1073.
55. Daigle SR, Olhava EJ, Therkelsen CA, et al. Selective killing of mixed lineage leukemia cells by a potent small-molecule DOT1L inhibitor. *Cancer Cell.* 2011;20(1):53-65.

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Selective inhibition of EZH2 and EZH1 enzymatic activity by a small molecule suppresses *MLL*-rearranged leukemia

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Polycomb genes, miRNA, and their deregulation in B-cell malignancies

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Posttranslational modifications of histone proteins represent a fundamental means to define distinctive epigenetic states and regulate gene expression during development and differentiation. Aberrations in various chromatin-modulation pathways are commonly used by tumors to initiate and maintain oncogenesis, including lymphomagenesis. Recently, increasing evidence has demonstrated that polycomb group (PcG) proteins, a subset of histone-modifying enzymes known to be crucial for B-cell maturation and differentiation, play a central role in malignant transformation of B cells. PcG hyperactivity in B-cell lymphomas is caused by overexpression or recurrent mutations of PcG genes and deregulation of microRNAs (miRNAs) or transcription factors such as c-MYC, which regulate PcG expression. Interplays of PcG and miRNA deregulations often establish a vicious signal-amplification loop in lymphoma associated with adverse clinical outcomes. Importantly, aberrant enzymatic activities associated with polycomb deregulation, notably those caused by EZH2 gain-of-function mutations, have provided a rationale for developing small-molecule inhibitors as novel therapies. In this review, we summarize our current understanding of PcG-mediated gene silencing, interplays of PcG with other epigenetic regulators such as miRNAs during B-cell differentiation and lymphomagenesis, and recent advancements in targeted strategies against PcG as promising therapeutics for B-cell malignancies. (*Blood*. 2015;125(8):1217-1225)

Introduction

Histone posttranslational modifications represent a fundamental mechanism for regulating DNA accessibility in various DNA-templated processes such as gene transcription.[1] Dysregulation of chromatin-modifying mechanisms is one of the central oncogenic pathways in human cancer,[1-3] including B-cell malignancies.[4-6]

Among various chromatin-modifying factors, polycomb group (PcG) proteins are critical for controlling gene expression, maintaining repressive chromatin states, and defining cellular identities during development.[7,8] PcG proteins act in multimeric complexes known as polycomb repressive complexes (PRCs). Two major PcG complexes exist in mammalian cells: PRC1 and PRC2. Biochemically, PRC1 employs an E3 ligase, RING1A or RING1B, to induce monoubiquitination of histone H2A, lysine 119 (H2AK119ub1) (Figure 1), a reaction that requires essential cofactors such as BMI1.[8] PRC2 utilizes an enzymatic subunit, enhancer of zeste homolog 2 (EZH2) or related EZH1, to methylate histone H3, lysine 27 (H3K27; Figure 1)[7]; other PRC2 subunits (EED and SUZ12) and accessory cofactors such as JARID2 and polycomb-like harbor either DNA- or histone-binding activities to modulate PRC2 activity and mediate its targeting or spreading on chromatin.[7-9] H2AK119ub1 and H3K27 trimethylation (H3K27me3) are prominent histone markers associated with gene silencing, indicating a causal role of PcG-mediated enzymatic activity in transcriptional regulation.[7,8] H3K27me3 also coexists with the gene-activation–associated trimethylation of histone H3, lysine 4 (H3K4me3) at "bivalent domain genes" to maintain genes in a repressed but poised conformation, which can be subsequently activated or stably repressed according to lineage-specific differentiation programs.[1]

In a simplistic hierarchical model, PRC2 acts upstream of PRC1 as H3K27me3 serves as a "docking" site for CBX, a chromodomain-containing protein (Figure 1A), which then recruits PRC1 to induce H2AK119ub1[7,8] (Figure 1B). However, more recently, data have demonstrated that PRC1 recruitment is both PRC2 dependent and PRC2 independent.[10,11] Furthermore, recent studies show that PRC1 can act upstream of PRC2. In this case, a PRC1 variant utilizes KDM2B, a CxxC-domain protein, to bind to the nonmethylated cytosine guanine dinucleotide sequence where PRC1-induced H2AK119ub1 recruits PRC2 via an unknown mechanism[12-14] (Figure 1C). EED, a PRC2 subunit, also physically interacts with PRC1, thus linking PRC2 to PRC1.[15] Overall, PRC2 and PRC1 cooperate and enforce gene silencing via positive-feedback loops.

Increasing evidence has revealed crucial roles of PcG proteins in myriad biological processes, including self-renewal, differentiation, cell-cycle control, senescence, and gene expression and imprinting,[7,8,16,17] all of which have been linked to oncogenesis when deregulated. Notably, *PcG* genes were found mutated in B-cell malignancies. B lymphoma Mo-MLV insertion region 1 homolog (*BMI1*, also known as polycomb group ring finger 4 or PCGF4 [Figure 1]) was originally isolated as a gene upregulated in murine B-cell lymphomas[18]; recurrent gain-of-function mutations of *EZH2* were identified in germinal center (GC) B-cell lymphomas.[4,19,20] Here, we focus on deregulations of PcG and cofactors during the initiation and development of B-cell malignancies. We also discuss the interplays between PcG and other epigenetic regulators such as microRNAs (miRNAs), histone deacetylases (HDACs), and DNA methytransferases (DNMTs). Finally, we summarize recent progress in development of PcG-specific inhibitors as novel therapies of B-cell malignancies.

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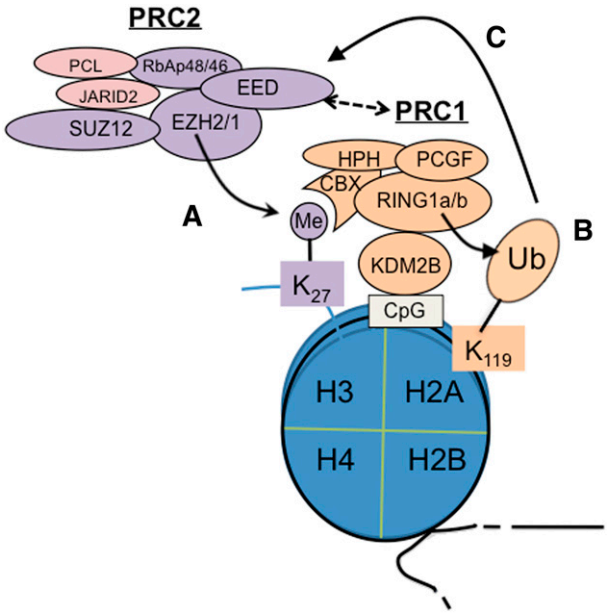

Figure 1. Cooperation of PRC2 and PRC1 in epigenetic silencing of genes. PRC2 catalyzes trimethylation of histone H3 at lysine 27 (H3K27me3) (A), which is recognized and bound by CBX proteins such as CBX7, a PRC1 subunit, to subsequently recruit PRC1 for induction of monoubiquitination of histone H2A at lysine 119 (H2AK119ub1)[7,8] (B).Conversely, recent studies show that a variant form of PRC1 can act upstream of PRC2 to initiate formation of the polycomb domain; in this case, H2AK119ub1 serves as a PRC2 recruitment mechanism (C).[12-14] In addition, EED is also shown to interact to PRC1 physically.[15] CpG, cytosine guanine dinucleotide; Me, trimethylation; Ub, ubiquitination.

Biological function of PcG proteins in B-cell development and lymphomagenesis

The development and differentiation of B-cell lineages initially occur with progenitor B-cell expansion and V(D)J gene rearrangement, a DNA recombination process that produces clonally unique, immunoglobulin variable regions for antigen recognition.[21] Upon antigen stimulation, B cells undergo activation through proliferation, somatic hypermutation, and antibody class switching, which occur in the GCs of secondary lymphoid tissues. A proliferative feature of GC B lymphocytes, with concomitant attenuation of their DNA damage repair function and ongoing somatic hypermutation, increases the likelihood of oncogenic mutation, genomic instability, and subsequent lymphomas. B-cell development is tightly controlled by genetic and epigenetic mechanisms, including DNA methylation, histone modification, chromatin remodeling,[22] and noncoding RNAs.[23] During normal B-lymphocyte differentiation, expression of PRC1 and PRC2 genes shows a restricted, stage-specific pattern. BMI1 and its PRC1 partners are primarily detected among resting B cells in the GC mantle zone and in nondividing centrocytes of the GC follicles; these PRC1 genes are silenced in proliferating follicular centroblasts, which then express the PRC2 genes instead.[24-26] In contrast, lymphomas generally lose such a mutually exclusive expression pattern, and altered expression of PRC1 and PRC2 genes is a general theme in lymphomas, including diffuse large B-cell lymphomas (DLBCL),[27] follicular lymphomas (FLs), and mantle cell lymphomas (MCLs).[28] These findings suggest essential regulatory roles of PRC1 and PRC2 in both normal B-lymphocyte development and lymphoma pathogenesis.

PRC1 in B-cell development and lymphomagenesis

BMI1

Bmi1 (also known as PCGF4 [Figure 1]) was initially discovered from a locus activated by viral integration in murine lymphomas.[18] BMI1 controls a range of B-cell developmental genes, including lineage master regulators *Ebf1* and *Pax5*.[29] *Bmi1* deficiency causes conversion of the "bivalent domain" states associated with *Ebf1* and *Pax5* to a monovalent active state, resulting in their premature expression and accelerated lymphoid differentiation.[29] BMI1 also directly represses expression of the tumor suppressors p16Ink4a/p19Arf and p15Ink4b; therefore, BMI1 overexpression prevents c-MYC–mediated apoptosis and was sufficient to induce lymphoma, a process further accelerated by *c-MYC*.[18,30] Furthermore, BMI1 represses the proapoptotic genes *Noxa* and *Bim*, supporting its pro-survival role in B-cell development and lymphomas.[31,32] In human B-cell lymphomas, BMI1 overexpression is common in almost all subtypes.[33] Expression of BMI1 alone or in combination with EZH2 characterizes aggressive B-cell lymphomas with unfavorable prognosis.[27,33,34] Recently, a novel t(10;14)(p12;q32) translocation was identified in chronic lymphocytic leukemia and MCLs leading to *IgH-BMI1* rearrangement and *BMI1* overexpression[35]; *IgH-BMI1* rearrangement was acquired during tumor high-grade transformation and correlated with chemoresistance.[35] Transcriptome analyses of multiple cancers found that BMI1-driven gene signatures define a phenotype of cancer stem cells,[36] suggesting that BMI1 confers malignant cells with features of cancer stem cells, the rare cancerous subpopulations that confer drug resistance and regeneration abilities.[16,37] Indeed, BMI1-mediated repression of p16Ink4a/p19Arf was shown to be essential for self-renewal of hematopoietic stem cells.[38] Overall, these studies support critical roles of BMI1 in promoting lymphoma progression and conferring therapy resistance.

Other PRC1 factors

Evidence exists showing direct involvement of other PRC1 components in B-cell lymphomas. CBX7 (Figure 1) was found highly expressed in GC lymphocytes and GC-derived FLs, where its elevated expression was correlated with *c-MYC* expression and an aggressive feature.[39] Lymphoid-specific overexpression of Cbx7 in mice initiated lymphomagenesis and cooperated with c-MYC to produce aggressive B-cell lymphomas.[39] Similar to BMI1, Cbx7 overexpression was linked to repression of p16Ink4a/p19Arf.[39] RING1A is associated with the risk of non-Hodgkin lymphomas[40] and high expression of RING1B detected in lymphomas such as DLBCLs and Burkitt lymphoma.[41] However, in the absence of p16Ink4a, RING1B deficiency accelerated lymphomagenesis through upregulation of cyclin D2 and Cdc6.[42] Thus, PRC1 harbors both oncogenic and tumor-suppressive roles in different contexts, which is reminiscent of PRC2's dual functions described among different hematopoietic malignancies.[43-45]

PRC2 in B-cell development and lymphomagenesis

EZH2 is highly expressed in lymphoid progenitors and required for efficient V(D)J recombination in pro–B cells.[46] *EZH2* is silenced

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in resting GC B cells but massively upregulated when GC B cells get activated and undergo rapid proliferation and immunoglobulin affinity maturation[26,47]; EZH2 blocks the DNA damage response pathways, allowing cells to survive the somatic hypermutation during antibody maturation.[26] Expression of *EZH2* strongly associates with B-cell malignancies, with its high levels correlated with the Ki67 labeling index, lymphoma aggressiveness, and unfavorable prognosis.[33,34] The highest percentage of EZH2 positivity was found in 100% of Burkitt lymphomas, 87.5% of grade-3 FLs, and 85.7% of DLBCLs. Multivariate survival analysis identified EZH2 as the strongest prognostic predictor of inferior outcomes of MCLs.[27] *SUZ12* expression was also found to be restricted to proliferating lymphoid cells during development and at high levels in MCLs, in comparison with its general absence in nontumorous mantle zone cells.[48]

The importance of PRC2 in lymphomagenesis is further strengthened by recent identification of recurrent missense mutations in EZH2, with the most prevalent ones altering a single residue in the catalytic domain, Y641 (the numeration of EZH2 amino acids based on a short isoform of EZH2 [National Center for Biotechnology Information accession Q15910.2]), among ~10% to 20% of GC-derived B-cell lymphomas such as DLBCLs and FLs.[4,5,49] These EZH2 mutations are likely to be early lesions during lymphomagenesis.[6,49] Biochemically, EZH2^{Y641} mutations alter substrate specificity of EZH2.[19,20,50] Being a catalytic subunit of PRC2, EZH2 induces sequential mono-, di-, and trimethylation of H3K27, with the highest methylation status most strongly associated with gene silencing.[51] Wild-type EZH2 has a greater catalytic efficiency for conducting monomethylation of H3K27 (H3K27me1) and a diminished efficiency for subsequent reactions (mono- to di- and di- to trimethylations).[19,20,50] In contrast, lymphoma-associated EZH2^{Y641X} mutations (X means Asn/Phe/Ser/His/Cys) show the exactly opposite substrate specificity, displaying limited ability to induce H3K27 monomethylation yet extremely high efficiency catalyzing the H3K27 di- to trimethylation reaction.[19,20,50] Such enzymatic differences between wild-type and EZH2^{Y641X} mutant protein suggest that EZH2^{Y641X} mutations must occur heterozygously in lymphomas, which is indeed the case in human patients,[4,5,49] allowing for EZH2^{Y641X} to cooperate with wild-type EZH2 to induce a global increase in H3K27me3[19,20,50] and aberrant transcriptional alteration. Later on, 2 additional somatic mutations, EZH2^{A677G} and EZH2^{A687V}, were identified at a lower frequency (~1% to 3%) among GC B-cell lymphomas,[5,49,52,53] and they demonstrate enzymatic properties distinct from EZH2^{Y641X} mutants.[50,52-54] EZH2^{A677G} and EZH2^{A687V} possess the almost equally enhanced catalytic activity towards all the H3K27 substrates with different methylation status.[50,52-54] Thus, EZH2^{A677G} and EZH2^{A687V} mutants are able to induce a global increase in H3K27me3 without the need for wild-type EZH2.[50] Besides kinetics, EZH2^{Y641X} also affects protein stability.[55] Phosphorylation of *Y641*, a known phosphorylation site of JAK2 kinases, leads to interaction of EZH2 with β-TrCP, a SCF E3 ubiquitin ligase, and promotes EZH2 degradation. Loss of this phosphorylation site due to somatic *Y641* mutations reduces EZH2 turnover, which has been postulated to contribute to the hyper-H3K27me3 phenotype.[55] Taken together, different gain-of-function mutations induce EZH2 hyperactivity through distinct molecular mechanisms, and despite the fact that lymphomas carrying different EZH2 mutations may possess different levels of the lowly methylated H3K27, they all have a consistently higher level of H3K27me3.[50,52-54]

Recent studies have provided a better understanding of the in vivo function of *EZH2* and mutation in normal B-cell development and lymphomagenesis.[47,56-58] Using *Ezh2* knockout mice, Beguelin et al[56] and Caganova et al[47] have independently shown that EZH2 is crucial for the formation of GCs and GC B-cell development. Mechanistically, EZH2 represses myriad downstream genes including the negative cell-cycle regulators *Cdkn2a* (p16Ink4a/p19Arf) and *Cdkn1a/p21* and crucial transcription factor genes IRF4 and BLIMP1/PRDM1, which are known to be essential for post GC B-cell development[47,56,57] (Figure 2). Indeed, depletion of EZH2 from lymphomas suppressed their proliferation and attenuated tumor formation.[57] These studies support the notion that EZH2 hyperactivation promotes malignant transformation by repressing both antiproliferative and differentiation-inducing programs. Furthermore, *Ezh2*-deficient GC B cells had profound impairments in GC responses and memory B-cell formation and failed to protect themselves from the genotoxic damages induced by activation-induced cytidinedeaminase,[47] an enzyme critical for somatic hypermutation and antibody affinity maturation,[21] demonstrating an essential role of EZH2 in the GC B-cell development (Figure 2). B-cell–specific expression of the EZH2^{Y641N} or EZH2^{Y641F} mutant in transgenic mice elevated the global H3K27me3, promoted a high proliferation of GC B cells, and resulted in follicular hyperplasia.[56,58] However, additional oncogenic events are required for neoplastic transformation, although GC-derived lymphomas remain addicted to EZH2 mutations. It has been shown that EZH2$^{Y641N/F}$ mutants cooperate with BCL2 to generate malignant GC B-cell lymphomas[56]; similarly, genetic interaction of EZH2^{Y641F} and MYC in transgenic mice gave rise to high-grade lymphomas with a mature B-cell phenotype.[58] Collectively, these findings have shown that dynamic expression of *EZH2* allows expansion and development of GC B cells, which undergo terminal differentiation and develop into antibody-secreting cells and plasma cells as *EZH2* expression declines (Figure 2). EZH2 hyperactivity perturbs the fine balance of GC B-cell proliferation and differentiation, permanently locking GC B cells in an immature and proliferative state, a prelude to full-blown lymphoma. Although not sufficient on its own to cause lymphoma, EZH2 gain-of-function mutations serve as a driver of lymphomagenesis and collaborate with additional lesions to generate and/or accelerate GC B-cell lymphomas (Figure 2).

In contrast to the oncogenic role of EZH2 in B-cell lineages, the tumor-suppressive roles of PRC2 in T-cell acute lymphoblastic leukemia[44,45] and myeloid malignancies[43] were identified due to a range of missense, nonsense, and frameshift mutations in EZH2, SUZ12, or EED (Figure 1). These lesions can be homozygous, are found throughout the gene, and are generally predicted to disable PRC2 activity, implying its disease-dependent functions. These observations emulate those obtained in Eμ-Myc lymphoma models showing that PRC2 can be a tumor suppressor in Eμ-Myc–induced lymphomagenesis, wherein the lymphoma onset was accelerated by knockdown of Suz12 or Ezh2.[59] Such an effect is likely due to the enhanced self-renewal of B-lymphoid progenitors upon PRC2 loss,[59] which is in contrast to cooperation between EZH2^{Y641F} and Eμ-Myc reported by Berg et al in GC B-cell compartments.[58] These studies emphasize the complicated, context-dependent role of PRC2 in oncogenesis, which might be due to a tightly controlled expression pattern of EZH2 throughout B-cell lineage differentiation. As a result, PRC2 activity at various developmental stages may either suppress or facilitate lymphomagenesis. Indeed, EZH2 expression is high in pro–B cells and decreased in pre–B cells and becomes nearly undetectable in immature naive cells[46,57]; EZH2 is then upregulated again during affinity maturation in GC B cells.[26,47] Therefore, it could be the case that whereas PRC2 restricts the proliferative and self-renewal potential of immature B-lymphoid progenitors, its gain-of-function mutations stimulate proliferation specifically in maturing

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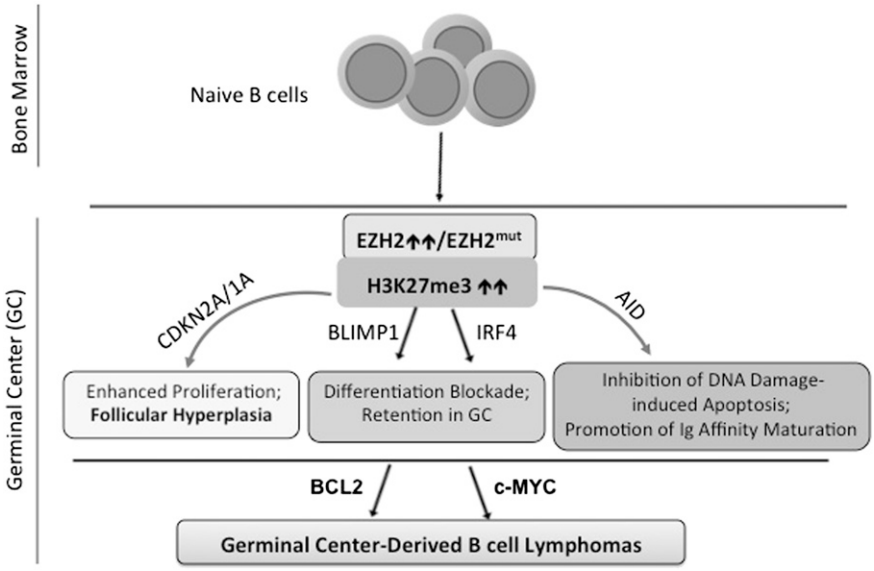

Figure 2. Biological functions of EZH2 in normal B-cell development and lymphomagenesis. During B-cell differentiation, naive B cells enter the GC and EZH2 is transcriptionally upregulated during GC B-cell maturation.[26,47] Via induction of H3K27me3, EZH2 then transcriptionally represses a myriad of downstream effector genes, which at least include the negative cell-cycle regulators (CDKN2A and CDKN1A) and B-cell differentiation-promoting transcription factors (IRF4 and BLIMP1/PRDM1), hence allowing for rapid expansion of immature B cells[47,56,57]; in addition, EZH2 protects GC B cells from the genotoxic damages induced by activation-induced cytidinedeaminase (AID),[47] an enzyme critical for immunoglobulin affinity maturation via a mechanism of somatic hypermutation that modifies the immunoglobulin variable region of the rearranged antibody genes in GC B cells.[21] EZH2 levels decrease as B cells exit the GC, enabling derepression of EZH2-targeted genes and hence terminal differentiation.[47,56,57] However, EZH2 hyperactivity (either somatic mutation or overexpression) disrupts such fine equilibrium, continuously enhances H3K27me3, and results in exaggerated silencing of EZH2 targeted genes, which then block GC B-cell differentiation and promote their proliferation and survival. EZH2 mutations alone lead to follicular hyperplasia, and, with acquisition of additional oncogenic events such as upregulation of BCL2 or c-MYC, EZH2 mutations cooperatively enable or accelerate malignant transformation of GC B cells.[56,58]

GC B cells. Further work with mouse models engineered to overexpress or delete EZH2 at each specific stage of B-cell differentiation shall provide insight into the role of PRC2 in various B-cell malignancies.

Interplay of PcG with other epigenetic enzymes

In addition to intrinsic enzymatic functions, PcG complexes also recruit other chromatin-modifying factors such as HDACs and DNMTs to re-enforce transcriptional repression. PRC2 recruits HDAC1-3, linking 2 distinctive repressive machineries together.[60] Several broad-spectrum HDAC inhibitors, including sodium butyrate, decrease the messenger RNA and protein levels of *BMI1* and *EZH2* in cancer cells.[61] These findings implicate that HDACs positively regulate cellular PcG levels and that epigenetic control of gene expression is governed by cooperation of PRC2 and HDACs. In addition, histone methylation influences DNA methylation and, in turn, DNA methylation serves as an instructive template for histone modification. In cancer, PcG-suppressed genes are likely to be associated with DNA hypermethylation, and hypermethylated promoters more frequently premarked with PcG.[8,62,63] Indeed, EZH2 directly associates with DNMTs.[64] This mechanism also appears to be operative in B-cell lymphoma because DNA methylation profiling of lymphomas revealed a significant enrichment of PcG targets at the de novo methylated genes,[65] indicating that crosstalk between histone and DNA methylation may form a double "locking" mechanism of an undifferentiated cell state during malignant transformation. Perturbation of cellular factors that antagonize PcG, such as trithorax group (TrxG) proteins,[17] may equally influence the regulatory roles and biological outputs of PcG complexes. Indeed, direct sequencing of patients with B-cell malignancies has recently led to identification of recurrent damaging mutations of several TrxG genes such as *MLL2*, *p300*, and *CBP*.[5,6,66] Loss-of-function mutations of TrxG and gain-of-function mutations of PcG genes may equally perturb a fine equilibrium of histone methylation dynamics during B-cell lymphomagenesis.

Interplay of PcG with miRNAs

miRNAs are 22-nucleotide, noncoding single-stranded RNAs that can repress gene expression at a posttranscriptional level. miRNAs are increasingly recognized as one of the major players in numerous biological processes, and their downderegulation is often seen in tumors, suggesting their tumor-suppressive roles. It can be anticipated that miRNA deregulation can contribute to PcG deregulation. Indeed, *EZH2* was the first PcG gene shown to be regulated by miRNA.[67,68] By targeting the 3′ untranslated region of *EZH2* messenger RNA, *miR-101* and *miR-26* repress cellular *EZH2* levels.[67,68] miRNAs that repress PRC1 genes were also identified.[69-71] Downregulation and deletion of these miRNAs are frequent in various tumors, including prostate cancer and lymphomas.[67,68] Conversely, PcG proteins also contribute to miRNA expression and deregulation during malignant development, given their frequent alterations found in tumors. Indeed, many miRNA genes are repressed by PRC2 and demarcated with H3K27me3.[72] PRC2 represses *miR-31* in adult T-cell lymphoma, leading to activation of nuclear factor κB oncogenic signaling.[73] Thus, these findings have shown an intriguing interplay between miRNAs and PcG. Below, we summarize recent advances in understanding their interactions in cancers, especially

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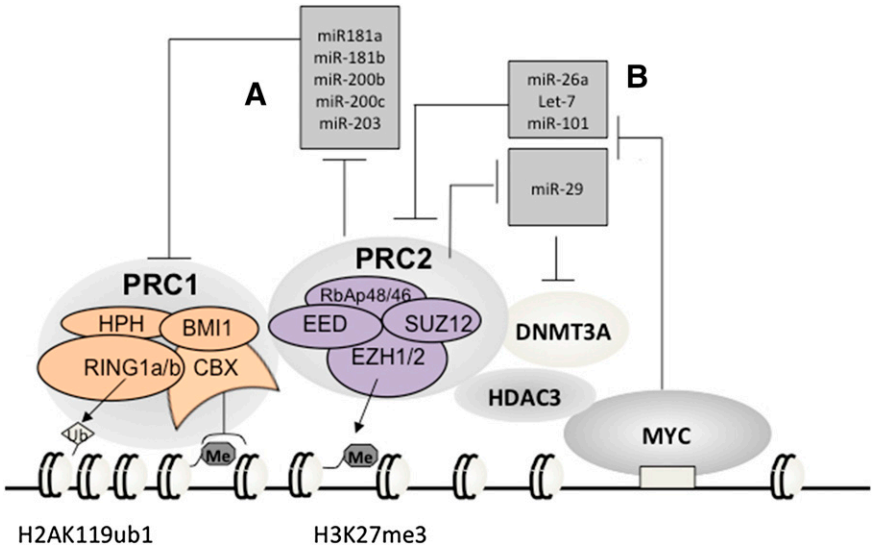

Figure 3. Vicious amplification loops involving a myriad of PcG proteins and miRNAs. Repression of PRC1-repressing miRNAs by PRC2 (A) establishes a positive-feedback loop ensuring coexpression and cooperation of 2 main PcG repressor complexes in stem and cancer cells; c-MYC, which is frequently translocated or overexpressed in Burkitt lymphoma and other B-cell lymphoma types, assembles a gene-silencing complex with PRC2 and HDACs to downregulate a list of tumor-suppressive miRNAs that can repress EZH2 and DNMT3A (B), hence establishing positive-feedback loops to enforce expression and functionality of PRC2 in B-cell lymphomas. Me3, trimethylation. Ub1, monoubiquitination.

B-cell malignancies, which reveal the hitherto-unappreciated regulatory circuits involving miRNA and epigenetic factors.

PRC2–miRNA–PRC1 circuitry

A subset of miRNAs, including miR-181a, miR-181b, miR-200b, miR-200c, and miR-203 (Figure 3A), are transcriptionally silenced by PRC2 in cancer.[69] Interestingly, these miRNAs repress PRC1 genes such as *BMI1* and RING1B[69-71] (Figure 3A). It has been shown that downregulation of these miRNAs such as miR-200c ensures the cellular level and functionality of PRC1 in stem cells[70,71] and cancers including lymphoma,[69-71,74] promoting cell "stemness" properties. These data demonstrate that expression of PRC1 and PRC2 is integrated through a network of regulatory miRNAs wherein epigenetic repression of PRC1-targeting miRNAs by PRC2 establishes a positive feedback loop, ensuring coexpression and cooperation of 2 major PcG complexes.

EZH2/c-MYC–miRNA–EZH2 circuitry

Recent studies of B-cell malignancies also unveiled a second circuitry involving EZH2, miRNAs, and c-MYC, an oncogenic transcription factor almost invariably translocated in Burkitt lymphoma. *c-MYC* assembles a repressive complex with PRC2 and HDACs to downregulate a broad spectrum of tumor-suppressive miRNAs, including miR-15a/16-1, miR-26, miR-27, miR-29, let-7, miR-494, and miR-548m[67,75-79] (Figure 3B). A similar c-MYC–PRC2 complex also represses miR-101 in hepatocellular carcinoma.[80] Among these repressed miRNAs, miR-101 and miR-26 were recurrently deleted in tumors including lymphoma[67,68]; miR-15a/16-1 targets BCL2 and acts as tumor suppressor in chronic lymphocytic leukemia.[81] Interestingly, several of these c-MYC–repressed miRNAs, including miR-26a, miR-101, and let-7, actually repress EZH2 directly[67,75,78] (Figure 3B); miR-29, a family of miRNAs known to be involved in B-cell lymphomagenesis,[75,82] was shown to downregulate DNMT3A, a PRC2-interacting factor, in chronic lymphocytic leukemia[83] (Figure 3B). Thus, via recruitment of PRC2 and HDACs, c-MYC, a prominent lymphoma-promoting factor, represses miRNAs that negatively regulate EZH2 and its cofactors, establishing a positive-feedback loop for enforcing polycomb genes expression and functionality in B-cell lymphomas. As knocking down EZH2 and HDACs led to re-expression of the MYC-repressed miRNAs,[75,78,84,85] the existing pharmacologic agents for inhibition of these c-MYC–associated corepressors shall represent a promising way to disrupt such a vicious amplification loop associated with lymphomagenesis.

Epigenetic therapy and perspective

Epigenetic deregulation of chromatin structure and function leads to aberrant gene expression and oncogenesis. Consequently, epigenetic therapies aim to restore normal chromatin-modification patterns through inhibition of the deregulated epigenetic machinery. HDAC and DNMT inhibitors are among the first promising agents for epigenetic therapies,[2] and, more recently, specific inhibitors for PcG proteins have been developed.

Targeting PRC1

A recent high-throughput screen discovered a small-molecule compound PTC-209 as an inhibitor of BMI1.[86] PTC-209 inhibited expression of *BMI1* and induced a dose-dependent reduction of global H2AK119ub1.[86] *BMI1* knockdown conferred PTC-209 insensitivity, indicating its specificity.[86] Similar to *BMI1* knockdown, PTC-209 treatment inhibited self-renewal of cancer-initiating stem cells.[86] This study implicates that BMI1 has the potential to be developed as a drug target for treating B-cell lymphomas with *BMI1* overexpression.

Targeting PRC2

Several highly selective small-molecule inhibitors of PRC2 (with K_i values within the low-nanomolar range) have recently been discovered,[87-93] many of which possess a common pyridone-containing motif that confers EZH2 or EZH1 inhibition (Figure 4A). Among them, EPZ005687[88] and GSK126[87] show high selectivity for EZH2 vs other methyltransferases, with >50- to 150-fold selectivity for EZH2 over EZH1 (Figure 4A). Early success was seen in treating B-cell lymphomas bearing EZH2 gain-of-function mutation with these inhibitors in DLBCL xenografts in mice.[56,87-93] GSK126,[87] EPZ005687, and EPZ-6438[88,94] (Figure 4A-B) show their particular effectiveness in suppressing growth of the EZH2-mutant lymphomas

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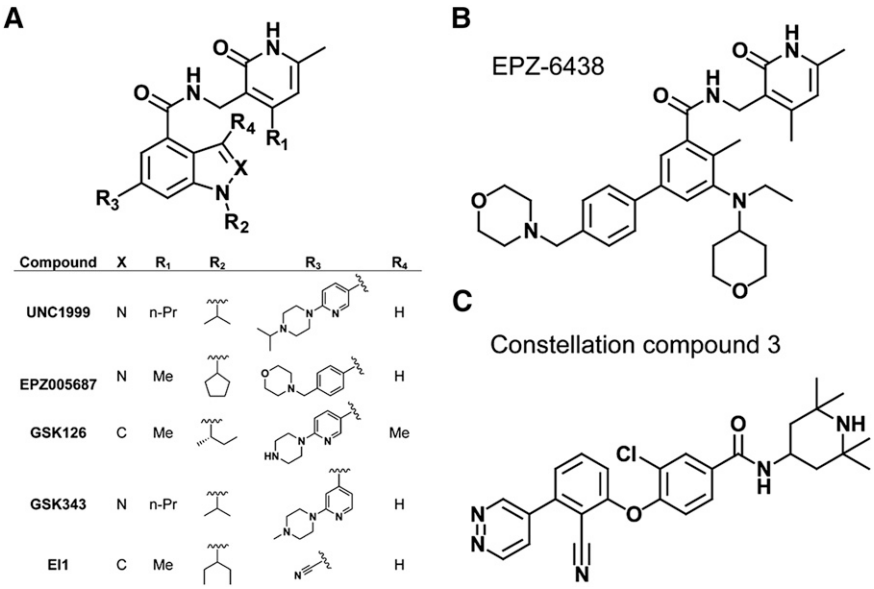

Figure 4. Highly selective, small-molecule inhibitors of PRC2. (A) Scaffold demonstrating that several of the recently developed EZH2 or EZH2/1 inhibitors all possess a pyridone motif as well as an indole or indazole core. The inserted table details the identity of each designated substituent of the described inhibitors. (B) Chemical structure of EPZ-6438. (C) Chemical structure of Constellation Pharmaceuticals compound 3, the first non–pyridone-containing EZH2 inhibitor.

vs those with wild-type EZH2. At the transcriptome level, and in contrast to minimal transcriptional responses in wild-type EZH2, drug-insensitive lines, reactivation of the formerly H3K27me3-demarcated genes was generally seen in drug-sensitive GC B-cell (GCB) type of DLBCL lines.[87,93] However, only a limited number of upregulated genes were found common across different inhibitor-sensitive lines, although upregulated genes in each individual line are enriched in those related to cell-cycle and apoptotic regulation,[87] thus highlighting a challenge to define relevant targets presumably due to variations of genetic backgrounds. The biological responses such as growth suppression after treatment with EZH2 inhibitors show a delayed pattern in comparison with the biochemical responses; the diminution of H3K27me3 was apparent within 24 to 48 hours post-treatment, and yet the cellular response is usually not fully presented until days 4 to 7 posttreatment and beyond.[87] Such a delayed effect with the PRC2 inhibitors was seen in acute myeloid leukemia[95] and for inhibitors of the histone methyltransferase DOT1L,[96] which is in contrast to quick responses associated with HDAC inhibitors. It has been speculated that additional time and/or chromatin factors may be required to reverse the histone methylation-regulated events, and, alternatively, catalytic inhibition induces compensatory recruitment of more enzymatic complexes at crucial gene targets, thus delaying demethylation. It is worth noting that PRC2 possesses noncanonical functions such as methylation of nonhistone substrates[97] or acting as transcriptional activator,[98] but it remains unclear if these mechanisms exist in lymphomas. Treatment of EZH2-mutant, GCB-DLBCL xenograft models with GSK126 or GSK343 (Figure 4A) resulted in tumor regression,[56,87] and the inhibitor was well tolerated. [87] Currently, various PRC2 inhibitors are under clinical evaluation and it would be exciting to see whether PRC2 inhibition provides clinical benefits for lymphoma patients.

EZH2 mutation ($EZH2^{Y641X}$, $EZH2^{A677G}$, or $EZH2^{A687V}$) is a known predictor of EZH2 inhibitor sensitivity; however, later studies showed that GCB-DLBCL is addicted to EZH2 and shows general sensitivity to the EZH2 inhibitor independent of its mutational state,[56] and such an EZH2 addiction was not seen in the activated B-cell type of DLBCLs, a more differentiated lymphoma subtype with EZH2 repressed.[56] These findings are consistent with the clinical observation that EZH2 mutations exclusively occur in GCB-DLBCLs and not activated B-cell DLBCL,[4] providing a rationale for a personalized medicine for lymphoma therapy. Furthermore, the efficacy of EZH2 inhibitors has been established in various B-cell lymphomas with wild-type EZH2, including MCLs and Burkitt lymphoma.[75,93] Therefore, other molecular determinants for EZH2 inhibitor sensitivity remain to be defined for B-cell lymphomas in order to improve personalized therapy. Such genetic determinants were defined in other cancers, including SNF inactivation in malignant rhabdoidtumors,[91] MMSET/NSD2 translocation in multiple myeloma,[99] and MLL rearrangement in acute leukemia,[95,100] and all of these affected pathways have been connected to PRC2 genetically. Thus, it is likely that lymphomas carrying TrxG gene mutations,[5,6,66] such as MLL2 mutations, may render sensitivity to PRC2 inhibition.

Furthermore, it remains to be examined if EZH1, a less-studied EZH2-related enzyme, is overexpressed in B-cell malignancies and if EZH1 inhibition improves the therapeutic potential. Given that EZH1 compensates the function of EZH2, inhibitors that target both EZH2 and EZH1 such as UNC1999[90] (Figure 4A) and Constellation Pharmaceuticals compound 3[92] (Figure 4C), are expected to have benefits for treating a broader spectrum of B-cell malignancies with overexpression of EZH2 or EZH1. Indeed, we have recently shown that UNC1999 offers advantages over EZH2-selective inhibitors and represents a novel efficient therapeutic for MLL-rearranged leukemias that coexpress EZH2 and EZH1.[95]

In line with therapeutic advances, combination therapy shall be explored, because EZH2 inhibitors can be used together with inhibitors against other oncogenic pathways that act in parallel. Indeed, our recent studies demonstrated synergy of EZH2 and HDAC inhibitors to inhibit lymphoma clonogenic cell growth, induce apoptosis, and suppress growth of MCLs or aggressive c-MYC–associated lymphomas.[75,77] The effects are at least partially due to disruption of c-MYC/EZH2-mediated miRNA silencing and vicious amplification loops (Figure 3B). In addition, combination treatment with EZH2 and BCL2 inhibitors outperformed the single-drug therapies in lymphoma models.[56] Lastly, given cooperation between DNA methylation and histone modification in transcriptional regulation, it would be of great interest to test if lymphoma cases with a higher degree of epigenetic silencing and hence reduced reversibility are less sensitive to PRC2 inhibitor single treatment but more responsive to a combined treatment with DNA demethylating agents.

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Conclusion

Gene regulation by PcG complexes is critical for regulation of various biological programs related to normal development and oncogenesis. PcG aberration, caused by its deregulated expression, somatic mutation, and chromosomal translocation, is common in various B-cell malignancies, demonstrating PcG as a central mechanism in lymphoma initiation and development. PcG complexes interact with other epigenetic machineries such as HDACs, DNMTs, and miRNAs in a context-dependent manner to control gene expression and promote lymphomagenesis. Recent advances in developing targeted strategies against PcG have demonstrated early success and display great potential in treating incurable B-cell malignancies.

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Authorship

Contribution: All authors participated in the preparation of the manuscript and illustrations and approved the final version of the manuscript.

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References

1. Chi P, Allis CD, Wang GG. Covalent histone modifications—miswritten, misinterpreted and mis-erased in human cancers. *Nat Rev Cancer.* 2010;10(7):457-469.
2. Arrowsmith CH, Bountra C, Fish PV, Lee K, Schapira M. Epigenetic protein families: a new frontier for drug discovery. *Nat Rev Drug Discov.* 2012;11(5):384-400.
3. Wang GG, Allis CD, Chi P. Chromatin remodeling and cancer, Part I: Covalent histone modifications. *Trends Mol Med.* 2007;13(9): 363-372.
4. Morin RD, Johnson NA, Severson TM, et al. Somatic mutations altering EZH2 (Tyr641) in follicular and diffuse large B-cell lymphomas of germinal-center origin. *Nat Genet.* 2010;42(2): 181-185.
5. Morin RD, Mendez-Lago M, Mungall AJ, et al. Frequent mutation of histone-modifying genes in non-Hodgkin lymphoma. *Nature.* 2011; 476(7360):298-303.
6. Okosun J, Bödör C, Wang J, et al. Integrated genomic analysis identifies recurrent mutations and evolution patterns driving the initiation and progression of follicular lymphoma. *Nat Genet.* 2014;46(2):176-181.
7. Margueron R, Reinberg D. The Polycomb complex PRC2 and its mark in life. *Nature.* 2011; 469(7330):343-349.
8. Di Croce L, Helin K. Transcriptional regulation by Polycomb group proteins. *Nat Struct Mol Biol.* 2013;20(10):1147-1155.
9. Cai L, Rothbart SB, Lu R, et al. An H3K36 methylation-engaging Tudor motif of polycomb-like proteins mediates PRC2 complex targeting. *Mol Cell.* 2013;49(3):571-582.
10. Tavares L, Dimitrova E, Oxley D, et al. RYBP-PRC1 complexes mediate H2A ubiquitylation at polycomb target sites independently of PRC2 and H3K27me3. *Cell.* 2012;148(4):664-678.
11. Leeb M, Pasini D, Novatchkova M, Jaritz M, Helin K, Wutz A. Polycomb complexes act redundantly to repress genomic repeats and genes. *Genes Dev.* 2010;24(3):265-276.
12. Cooper S, Dienstbier M, Hassan R, et al. Targeting polycomb to pericentric heterochromatin in embryonic stem cells reveals a role for H2AK119u1 in PRC2 recruitment. *Cell Reports.* 2014;7(5):1456-1470.
13. Blackledge NP, Farcas AM, Kondo T, et al. Variant PRC1 complex-dependent H2A ubiquitylation drives PRC2 recruitment and polycomb domain formation. *Cell.* 2014;157(6): 1445-1459.
14. Kalb R, Latwiel S, Baymaz HI, et al. Histone H2A monoubiquitination promotes histone H3 methylation in Polycomb repression. *Nat Struct Mol Biol.* 2014;21(6):569-571.
15. Cao Q, Wang X, Zhao M, et al. The central role of EED in the orchestration of polycomb group complexes. *Nat Commun.* 2014;5:3127.
16. Bracken AP, Helin K. Polycomb group proteins: navigators of lineage pathways led astray in cancer. *Nat Rev Cancer.* 2009;9(11):773-784.
17. Schuettengruber B, Chourrout D, Vervoort M, Leblanc B, Cavalli G. Genome regulation by polycomb and trithorax proteins. *Cell.* 2007; 128(4):735-745.
18. van Lohuizen M, Verbeek S, Scheijen B, Wientjens E, van der Gulden H, Berns A. Identification of cooperating oncogenes in E mu-myc transgenic mice by provirus tagging. *Cell.* 1991;65(5):737-752.
19. Sneeringer CJ, Scott MP, Kuntz KW, et al. Coordinated activities of wild-type plus mutant EZH2 drive tumor-associated hypertrimethylation of lysine 27 on histone H3 (H3K27) in human B-cell lymphomas. *Proc Natl Acad Sci USA.* 2010;107(49):20980-20985.
20. Yap DB, Chu J, Berg T, et al. Somatic mutations at EZH2 Y641 act dominantly through a mechanism of selectively altered PRC2 catalytic activity, to increase H3K27 trimethylation. *Blood.* 2011;117(8):2451-2459.
21. Klein U, Dalla-Favera R. Germinal centres: role in B-cell physiology and malignancy. *Nat Rev Immunol.* 2008;8(1):22-33.
22. Wang GG, Allis CD, Chi P. Chromatin remodeling and cancer, Part II: ATP-dependent chromatin remodeling. *Trends Mol Med.* 2007; 13(9):373-380.
23. Schmidt A, Küppers R. Role of microRNAs in B cell leukemias and lymphomas. *Curr Mol Med.* 2014;14(5):580-597.
24. Raaphorst FM, van Kemenade FJ, Fieret E, et al. Cutting edge: polycomb gene expression patterns reflect distinct B cell differentiation stages in human germinal centers. *J Immunol.* 2000;164(1):1-4.
25. Raaphorst FM, Otte AP, van Kemenade FJ, et al. Distinct BMI-1 and EZH2 expression patterns in thymocytes and mature T cells suggest a role for Polycomb genes in human T cell differentiation. *J Immunol.* 2001;166(10): 5925-5934.
26. van Galen JC, Dukers DF, Giroth C, et al. Distinct expression patterns of polycomb oncoproteins and their binding partners during the germinal center reaction. *Eur J Immunol.* 2004;34(7):1870-1881.
27. van Kemenade FJ, Raaphorst FM, Blokzijl T, et al. Coexpression of BMI-1 and EZH2 polycomb-group proteins is associated with cycling cells and degree of malignancy in B-cell non-Hodgkin lymphoma. *Blood.* 2001;97(12): 3896-3901.
28. Visser HP, Gunster MJ, Kluin-Nelemans HC, et al. The Polycomb group protein EZH2 is upregulated in proliferating, cultured human mantle cell lymphoma. *Br J Haematol.* 2001; 112(4):950-958.
29. Oguro H, Yuan J, Ichikawa H, et al. Poised lineage specification in multipotential hematopoietic stem and progenitor cells by the polycomb protein Bmi1. *Cell Stem Cell.* 2010; 6(3):279-286.
30. Jacobs JJ, Scheijen B, Voncken JW, Kieboom K, Berns A, van Lohuizen M. Bmi-1 collaborates with c-Myc in tumorigenesis by inhibiting c-Myc-induced apoptosis via INK4a/ARF. *Genes Dev.* 1999;13(20):2678-2690.

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31. Yamashita M, Kuwahara M, Suzuki A, et al. Bmi1 regulates memory CD4 T cell survival via repression of the Noxa gene. *J Exp Med.* 2008; 205(5):1109-1120.
32. Jagani Z, Wiederschain D, Loo A, et al. The Polycomb group protein Bmi-1 is essential for the growth of multiple myeloma cells. *Cancer Res.* 2010;70(13):5528-5538.
33. Abd Al Kader L, Oka T, Takata K, et al. In aggressive variants of non-Hodgkin lymphomas, Ezh2 is strongly expressed and polycomb repressive complex PRC1.4 dominates over PRC1.2. *Virchows Arch.* 2013;463(5):697-711.
34. van Galen JC, Muris JJ, Oudejans JJ, et al. Expression of the polycomb-group gene BMI1 is related to an unfavourable prognosis in primary nodal DLBCL. *J Clin Pathol.* 2007;60(2): 167-172.
35. Rouhigharabaei L, Ferreiro JF, Put N, et al. BMI1, the polycomb-group gene, is recurrently targeted by genomic rearrangements in progressive B-cell leukemia/lymphoma. *Genes Chromosomes Cancer.* 2013;52(10):928-944.
36. Glinsky GV, Berezovska O, Glinskii AB. Microarray analysis identifies a death-from-cancer signature predicting therapy failure in patients with multiple types of cancer. *J Clin Invest.* 2005;115(6):1503-1521.
37. Lessard J, Sauvageau G. Bmi-1 determines the proliferative capacity of normal and leukaemic stem cells. *Nature.* 2003;423(6937):255-260.
38. Park IK, Qian D, Kiel M, et al. Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. *Nature.* 2003; 423(6937):302-305.
39. Scott CL, Gil J, Hernando E, et al. Role of the chromobox protein CBX7 in lymphomagenesis. *Proc Natl Acad Sci USA.* 2007;104(13): 5389-5394.
40. Wang SS, Menashe I, Cerhan JR, et al. Variations in chromosomes 9 and 6p21.3 with risk of non-Hodgkin lymphoma. *Cancer Epidemiol Biomarkers Prev.* 2011;20(1):42-49.
41. Sánchez-Beato M, Sánchez E, González-Carreró J, et al. Variability in the expression of polycomb proteins in different normal and tumoral tissues. A pilot study using tissue microarrays. *Mod Pathol.* 2006;19(5):684-694.
42. Calés C, Román-Trufero M, Pavón L, et al. Inactivation of the polycomb group protein Ring1B unveils an antiproliferative role in hematopoietic cell expansion and cooperation with tumorigenesis associated with Ink4a deletion. *Mol Cell Biol.* 2008;28(3):1018-1028.
43. Shih AH, Abdel-Wahab O, Patel JP, Levine RL. The role of mutations in epigenetic regulators in myeloid malignancies. *Nat Rev Cancer.* 2012; 12(9):599-612.
44. Simon C, Chagraoui J, Krosl J, et al. A key role for EZH2 and associated genes in mouse and human adult T-cell acute leukemia. *Genes Dev.* 2012;26(7):651-656.
45. Ntziachristos P, Tsirigos A, Van Vlierberghe P, et al. Genetic inactivation of the polycomb repressive complex 2 in T cell acute lymphoblastic leukemia. *Nat Med.* 2012;18(2): 298-301.
46. Su IH, Basavaraj A, Krutchinsky AN, et al. Ezh2 controls B cell development through histone H3 methylation and Igh rearrangement. *Nat Immunol.* 2003;4(2):124-131.
47. Caganova M, Carrisi C, Varano G, et al. Germinal center dysregulation by histone methyltransferase EZH2 promotes lymphomagenesis. *J Clin Invest.* 2013;123(12): 5009-5022.
48. Martín-Pérez D, Sánchez E, Maestre L, et al. Deregulated expression of the polycomb-group protein SUZ12 target genes characterizes mantle cell lymphoma. *Am J Pathol.* 2010; 177(2):930-942.
49. Bödör C, Grossmann V, Popov N, et al. EZH2 mutations are frequent and represent an early event in follicular lymphoma. *Blood.* 2013; 122(18):3165-3168.
50. Swalm BM, Knutson SK, Warholic NM, et al. Reaction coupling between wild-type and disease-associated mutant EZH2. *ACS Chem Biol.* 2014;9(11):2459-2464.
51. Barski A, Cuddapah S, Cui K, et al. High-resolution profiling of histone methylations in the human genome. *Cell.* 2007;129(4):823-837.
52. Majer CR, Jin L, Scott MP, et al. A687V EZH2 is a gain-of-function mutation found in lymphoma patients. *FEBS Lett.* 2012;586(19):3448-3451.
53. McCabe MT, Graves AP, Ganji G, et al. Mutation of A677 in histone methyltransferase EZH2 in human B-cell lymphoma promotes hypertrimethylation of histone H3 on lysine 27 (H3K27). *Proc Natl Acad Sci USA.* 2012;109(8): 2989-2994.
54. Ott HM, Graves AP, Pappalardi MB, et al. A687V EZH2 Is a Driver of Histone H3 Lysine 27 (H3K27) Hypertrimethylation. *Mol Cancer Ther.* 2014;13(12):3062-3073.
55. Sahasrabuddhe AA, Chen X, Chung F, Velusamy T, Lim MS, Elenitoba-Johnson KS. Oncogenic Y641 mutations in EZH2 prevent Jak2/β-TrCP-mediated degradation [published online ahead of print January 27, 2014]. *Oncogene.*
56. Béguelin W, Popovic R, Teater M, et al. EZH2 is required for germinal center formation and somatic EZH2 mutations promote lymphoid transformation. *Cancer Cell.* 2013;23(5): 677-692.
57. Velichutina I, Shaknovich R, Geng H, et al. EZH2-mediated epigenetic silencing in germinal center B cells contributes to proliferation and lymphomagenesis. *Blood.* 2010;116(24): 5247-5255.
58. Berg T, Thoene S, Yap D, et al. A transgenic mouse model demonstrating the oncogenic role of mutations in the polycomb-group gene EZH2 in lymphomagenesis. *Blood.* 2014;123(25): 3914-3924.
59. Lee SC, Phipson B, Hyland CD, et al. Polycomb repressive complex 2 (PRC2) suppresses Eμ-myc lymphoma. *Blood.* 2013;122(15): 2654-2663.
60. van der Vlag J, Otte AP. Transcriptional repression mediated by the human polycomb-group protein EED involves histone deacetylation. *Nat Genet.* 1999;23(4):474-478.
61. Fiskus W, Wang Y, Sreekumar A, et al. Combined epigenetic therapy with the histone methyltransferase EZH2 inhibitor 3-deazaneplanocin A and the histone deacetylase inhibitor panobinostat against human AML cells. *Blood.* 2009;114(13):2733-2743.
62. Ohm JE, McGarvey KM, Yu X, et al. A stem cell-like chromatin pattern may predispose tumor suppressor genes to DNA hypermethylation and heritable silencing. *Nat Genet.* 2007;39(2): 237-242.
63. Schlesinger Y, Straussman R, Keshet I, et al. Polycomb-mediated methylation on Lys27 of histone H3 pre-marks genes for de novo methylation in cancer. *Nat Genet.* 2007;39(2): 232-236.
64. Viré E, Brenner C, Deplus R, et al. The Polycomb group protein EZH2 directly controls DNA methylation. *Nature.* 2006;439(7078): 871-874.
65. Martín-Subero JI, Kreuz M, Bibikova M, et al; Molecular Mechanisms in Malignant Lymphomas Network Project of the Deutsche Krebshilfe. New insights into the biology and origin of mature aggressive B-cell lymphomas by combined epigenomic, genomic, and transcriptional profiling. *Blood.* 2009;113(11):2488-2497.
66. Mullighan CG, Zhang J, Kasper LH, et al. CREBBP mutations in relapsed acute lymphoblastic leukaemia. *Nature.* 2011; 471(7337):235-239.
67. Sander S, Bullinger L, Klapproth K, et al. MYC stimulates EZH2 expression by repression of its negative regulator miR-26a. *Blood.* 2008; 112(10):4202-4212.
68. Varambally S, Cao Q, Mani RS, et al. Genomic loss of microRNA-101 leads to overexpression of histone methyltransferase EZH2 in cancer. *Science.* 2008;322(5908):1695-1699.
69. Cao Q, Mani RS, Ateeq B, et al. Coordinated regulation of polycomb group complexes through microRNAs in cancer. *Cancer Cell.* 2011;20(2): 187-199.
70. Shimono Y, Zabala M, Cho RW, et al. Downregulation of miRNA-200c links breast cancer stem cells with normal stem cells. *Cell.* 2009;138(3):592-603.
71. Wellner U, Schubert J, Burk UC, et al. The EMT-activator ZEB1 promotes tumorigenicity by repressing stemness-inhibiting microRNAs. *Nat Cell Biol.* 2009;11(12):1487-1495.
72. Marson A, Levine SS, Cole MF, et al. Connecting microRNA genes to the core transcriptional regulatory circuitry of embryonic stem cells. *Cell.* 2008;134(3):521-533.
73. Yamagishi M, Nakano K, Miyake A, et al. Polycomb-mediated loss of miR-31 activates NIK-dependent NF-κB pathway in adult T cell leukemia and other cancers. *Cancer Cell.* 2012; 21(1):121-135.
74. Cui J, Cheng Y, Zhang P, et al. Down regulation of miR200c promotes radiation-induced thymic lymphoma by targeting BMI1. *J Cell Biochem.* 2014;115(6):1033-1042.
75. Zhang X, Zhao X, Fiskus W, et al. Coordinated silencing of MYC-mediated miR-29 by HDAC3 and EZH2 as a therapeutic target of histone modification in aggressive B-Cell lymphomas. *Cancer Cell.* 2012;22(4):506-523.
76. Zhang X, Chen X, Lin J, et al. Myc represses miR-15a/miR-16-1 expression through recruitment of HDAC3 in mantle cell and other non-Hodgkin B-cell lymphomas. *Oncogene.* 2012;31(24):3002-3008.
77. Zhao X, Lwin T, Zhang X, et al. Disruption of the MYC-miRNA-EZH2 loop to suppress aggressive B-cell lymphoma survival and clonogenicity. *Leukemia.* 2013;27(12):2341-2350.
78. Tao J, Zhao X, Tao J. c-MYC-miRNA circuitry: a central regulator of aggressive B-cell malignancies. *Cell Cycle.* 2014;13(2):191-198.
79. Chang TC, Yu D, Lee YS, et al. Widespread microRNA repression by Myc contributes to tumorigenesis. *Nat Genet.* 2008;40(1):43-50.
80. Wang L, Zhang X, Jia LT, et al. c-Myc-mediated epigenetic silencing of MicroRNA-101 contributes to dysregulation of multiple pathways in hepatocellular carcinoma. *Hepatology.* 2014; 59(5):1850-1863.
81. Cimmino A, Calin GA, Fabbri M, et al. miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc Natl Acad Sci USA.* 2005;102(39): 13944-13949.
82. Zhao JJ, Lin J, Lwin T, et al. microRNA expression profile and identification of miR-29 as a prognostic marker and pathogenetic factor by targeting CDK6 in mantle cell lymphoma. *Blood.* 2010;115(13):2630-2639.
83. Santanam U, Zanesi N, Efanov A, et al. Chronic lymphocytic leukemia modeled in mouse by

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targeted miR-29 expression. *Proc Natl Acad Sci USA.* 2010;107(27):12210-12215.

84. Lwin T, Zhao X, Cheng F, et al. A microenvironment-mediated c-Myc/miR-548m/HDAC6 amplification loop in non-Hodgkin B cell lymphomas. *J Clin Invest.* 2013;123(11):4612-4626.
85. Ferreira AC, Robaina MC, Rezende LM, Severino P, Klumb CE. Histone deacetylase inhibitor prevents cell growth in Burkitt's lymphoma by regulating PI3K/Akt pathways and leads to upregulation of miR-143, miR-145, and miR-101. *Ann Hematol.* 2014;93(6):983-993.
86. Kreso A, van Galen P, Pedley NM, et al. Self-renewal as a therapeutic target in human colorectal cancer. *Nat Med.* 2014;20(1):29-36.
87. McCabe MT, Ott HM, Ganji G, et al. EZH2 inhibition as a therapeutic strategy for lymphoma with EZH2-activating mutations. *Nature.* 2012;492(7427):108-112.
88. Knutson SK, Wigle TJ, Warholic NM, et al. A selective inhibitor of EZH2 blocks H3K27 methylation and kills mutant lymphoma cells. *Nat Chem Biol.* 2012;8(11):890-896.
89. Qi W, Chan H, Teng L, et al. Selective inhibition of Ezh2 by a small molecule inhibitor blocks tumor cells proliferation. *Proc Natl Acad Sci USA.* 2012;109(52):21360-21365.
90. Konze KD, Ma A, Li F, et al. An orally bioavailable chemical probe of the Lysine Methyltransferases EZH2 and EZH1. *ACS Chem Biol.* 2013;8(6):1324-1334.
91. Knutson SK, Warholic NM, Wigle TJ, et al. Durable tumor regression in genetically altered malignant rhabdoid tumors by inhibition of methyltransferase EZH2. *Proc Natl Acad Sci USA.* 2013;110(19):7922-7927.
92. Garapaty-Rao S, Nasveschuk C, Gagnon A, et al. Identification of EZH2 and EZH1 small molecule inhibitors with selective impact on diffuse large B cell lymphoma cell growth. *Chem Biol.* 2013;20(11):1329-1339.
93. Bradley WD, Arora S, Busby J, et al. EZH2 Inhibitor Efficacy in Non-Hodgkin's Lymphoma Does Not Require Suppression of H3K27 Monomethylation. *Chem Biol.* 2014;21(11):1463-1475.
94. Knutson SK, Kawano S, Minoshima Y, et al. Selective inhibition of EZH2 by EPZ-6438 leads to potent antitumor activity in EZH2-mutant non-Hodgkin lymphoma. *Mol Cancer Ther.* 2014;13(4):842-854.
95. Xu B, On DM, Ma A, et al. Selective inhibition of EZH2 and EZH1 enzymatic activity by a small molecule suppresses MLL-rearranged leukemia [published online ahead of print November 13, 2014]. *Blood.* 2015;125(2):346-357.
96. Daigle SR, Olhava EJ, Therkelsen CA, et al. Selective killing of mixed lineage leukemia cells by a potent small-molecule DOT1L inhibitor. *Cancer Cell.* 2011;20(1):53-65.
97. Kim E, Kim M, Woo DH, et al. Phosphorylation of EZH2 activates STAT3 signaling via STAT3 methylation and promotes tumorigenicity of glioblastoma stem-like cells. *Cancer Cell.* 2013;23(6):839-852.
98. Xu K, Wu ZJ, Groner AC, et al. EZH2 oncogenic activity in castration-resistant prostate cancer cells is Polycomb-independent. *Science.* 2012;338(6113):1465-1469.
99. Popovic R, Martinez-Garcia E, Giannopoulou EG, et al. Histone methyltransferase MMSET/NSD2 alters EZH2 binding and reprograms the myeloma epigenome through global and focal changes in H3K36 and H3K27 methylation. *PLoS Genet.* 2014;10(9):e1004566.
100. Neff T, Sinha AU, Kluk MJ, et al. Polycomb repressive complex 2 is required for MLL-AF9 leukemia. *Proc Natl Acad Sci USA.* 2012;109(13):5028-5033.

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Polycomb genes, miRNA, and their deregulation in B-cell malignancies

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Targeting EZH2 and PRC2 dependence as novel anticancer therapy

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Distinctive patterns of chromatin modification control gene expression and define cellular identity during development and cell differentiation. Polycomb repressive complex 2 (PRC2), the sole mammalian enzymatic complex capable of establishing gene-repressive high-degree methylation of histone H3 at lysine 27 (H3K27), plays crucial roles in regulation of normal and malignant hematopoiesis. Recently, increasing evidence has indicated that recurrent gain-of-function mutation and overexpression of EZH2, the catalytic subunit of PRC2, drive and promote malignant transformation such as B-cell lymphomagenesis, providing a rationale for PRC2 inhibition as a novel anticancer strategy. Here, we summarize the recently developed strategies for inhibition of PRC2, which include a series of highly specific, highly potent, small-molecule inhibitors of EZH2 and EZH1, an EZH2-related methyltransferase. PRC2 establishes functional crosstalk with numerous epigenetic machineries during dynamic regulation of gene transcription. Perturbation of such functional crosstalk caused by genetic events observed in various hematologic cancers, such as inactivation of *SNF5* and somatic mutation of *UTX*, confers PRC2 dependence, thus rendering an increased sensitivity to PRC2 inhibition. We discuss our current understanding of EZH2 somatic mutations frequently found in B-cell lymphomas and recurrent mutations in various other epigenetic regulators as novel molecular predictors and determinants of PRC2 sensitivity. As recent advances have indicated a critical developmental or tumor-suppressive role for PRC2 and EZH2 in various tissue types, we discuss concerns over potentially toxic or even adverse effects associated with EZH2/1 inhibition in certain biological contexts or on cancer genetic background. Collectively, inhibition of PRC2 catalytic activity has emerged as a promising therapeutic intervention for the precise treatment of a range of genetically defined hematologic malignancies and can be potentially applied to a broader spectrum of human cancers that bear similar genetic and epigenetic characteristics. **Copyright © 2015 ISEH - International Society for Experimental Hematology. Published by Elsevier Inc.**

Chromatin modification provides a fundamental means for regulating DNA accessibility in a wide range of DNA-templated processes such as gene transcription and DNA damage repair [1]. At the very least, the regulatory mechanisms for defining distinctive chromatin states include DNA methylation [2], posttranslational modification of histones [3], ATP-dependent chromatin remodeling [4], and utility of histone variants [5]. Deregulation of these chromatin regulatory mechanisms is directly involved in oncogenesis, including hematopoietic malignancies [3,4,6–9].

Polycomb repressive complex 2 (PRC2) probably represents the only enzymatic machinery capable of catalyzing the high-level methylation of histone H3 at lysine 27 (H3K27me3/2) in mammalian cells [10,11]. H3K27 trimethylation (H3K27me3) strongly associates with transcriptional repression [10,11]. Extensive studies have indicated that PRC2 and, by extension, H3K27me3 play critical roles in cell fate determination during development including hematopoietic lineage specification [12–15]. Biochemically, PRC2 uses either enhancer of zeste homologue 2 (EZH2) or a related homolog (EZH1) as the catalytic subunit, and other core components such as EED and SUZ12 are requisite for forming a stable and enzymatically active methyltransferase complex [10,11]. The DNA- and histone-binding properties harbored by PRC2 core components [10,11] and several PRC2-associated proteins [16–20]

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or noncoding RNAs [21] have been found to regulate PRC2's target specificity and mediate its chromatin association and/or spreading on chromatin. Hyperactivation of PRC2 caused by mutation or overexpression of EZH2 has been identified as a frequent genetic event among B-cell lymphomas [22–24]. In the next sections, we focus on our current understanding of the lymphoma-associated EZH2 mutations and recent exciting advances in developing EZH2/1-specific inhibitors as a novel molecularly targeted therapy. Because of the high degree of crosstalk between PRC2 and other chromatin regulatory machineries, we also cover recent findings of novel molecular determinants yielding sensitivity to PRC2 inhibition and discuss the potential applications for small-molecule PRC2 inhibitors as therapies in several genetically defined hematologic cancers.

Lymphoma-associated gain-of-function mutation of *EZH2*

During germinal-center (GC) B-cell development, EZH2 exhibits a dynamic expression pattern: it is massively upregulated when B cells undergo rapid proliferation and immunoglobulin affinity maturation on immune activation, and decreased after completion of these processes [14,25], suggesting a key role for EZH2 in GC development. EZH2 is expressed in a wide range of B-cell neoplasms including Burkitt's lymphoma, mantle cell lymphomas (MCLs), follicular lymphoma (FL), and diffuse large B-cell lymphomas (DLBCLs) [26,27]. It is upregulated in MCLs as compared with the normal tissue counterparts from which the lymphoma derived [28]. EZH2 deregulation indeed associates with B-cell lymphomagenesis [26], with its expression levels positively correlated with aggressiveness and unfavorable prognosis [29,30]. The importance of PRC2 in lymphomagenesis is further strengthened by the recent identification of recurrent, heterozygous missense mutations of *EZH2* in B-cell lymphomas of GC origin such as DLBCLs and FLs. These mutations specifically target the catalytic SET domain of EZH2. The most prevalent is a point mutation of the Tyr641 residue found mutated to either Asn, Phe, Cys, Ser, or His (Fig. 1A, the numeration of EZH2 amino acids based on a short isoform [NCBI Accession No. Q15910.2]) in ~10%–20% of DLBCLs and FLs [22,31,32]; two other rare EZH2 mutations, A677G and A687V (Fig. 1A), were reported in about ~1%–3% of B-cell lymphoma cases [23,31–33].

Biochemically, lymphoma-associated *EZH2* mutations alter substrate specificity of the PRC2 complex [31,34,35]. EZH2 can induce mono-, di-, and trimethylation of H3K27 (i.e., H3K27me1/2/3), with H3K27me3/2 most strongly associated with gene silencing. Kinetic studies in vitro indicate that PRC2 complexes assembled by the wild-type EZH2 (i.e., PRC2-EZH2^{WT}) have the greatest

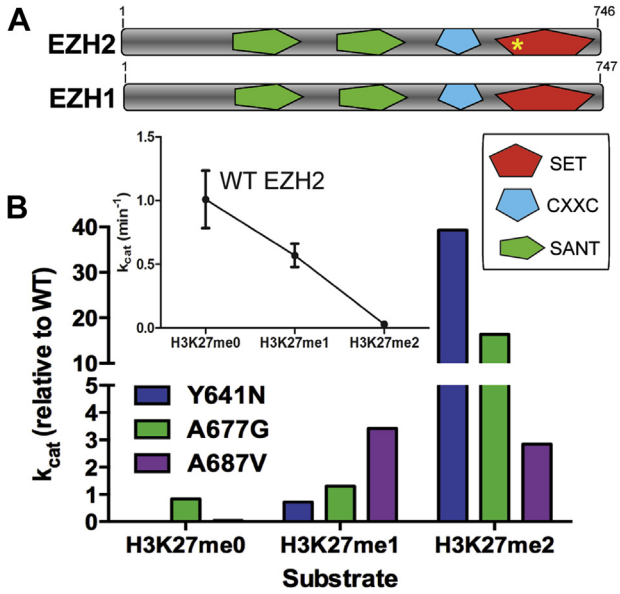

Figure 1. Gain-of-function EZH2 mutations affect substrate specificity of the PRC2 complex. (**A**) Depiction of EZH2 and EZH1 domain structure with the site of gain-of-function mutations (either the hotspot Y641 mutation, A667G, or A687V) in the catalytic SET domain of EZH2 highlighted with a *yellow asterisk*. (**B**) Wild-type EZH2 is more efficient at catalyzing the turnover of H3K27me0 and H3K27me1 than H3K27me2 (shown in inset, *black line*). Y641N (*blue bars*) and A677G (*green bars*) exhibit the opposite trend. A687V (*purple bars*) is equipotent at catalyzing the turnover of H3K27me1 and H3K27me2, relative to WT EZH2. *WT* = wild-type.

catalytic efficiency for converting nonmethylated H3K27 (H3K27me0) to monomethylated H3K27 (H3K27me1) and exhibit diminished efficiency for subsequent (H3K27me1 to H3K27me2 and H3K27me2 to H3K27me3) reactions [31,34,35] (Fig. 1B, inset). In contrast, PRC2 complexes bearing a lymphoma-associated EZH2 Tyr641 hotspot mutation such as Y641N (i.e., PRC2-EZH2^{Y641N}) display very limited ability to methylate nonmethylated H3K27, but once H3K27 is monomethylated, they can catalyze the turnover of H3K27me1 to H3K27me2 and, then, much more rapidly catalyze the H3K27me2-to-H3K27me3 reaction [31,34,35] (Fig. 1B, *blue bars*). The enzymatic differences between PRC2-EZH2^{WT} and PRC2-EZH2^{Y641N} require cooperation to produce the abnormally high level of H3K27me3 seen in the lymphoma cells [31,34,35]; a similar phenomenon was observed for other Tyr641 mutations such as Y641F [31,34,35]. Two other rare EZH2 SET domain mutations, A677G and A687V (Fig. 1A), also affect the substrate specificity of PRC2; however, they have kinetic properties that differ from those of the Y641 mutants [31,32,36,37]. For example, the A677G mutation leads to a form of EZH2 that is capable of efficiently methylating all the H3K27 substrates (Fig. 1B, *green bars*). Contrarily, the A687V mutation yields a PRC2 complex that is equipotent at methylating H3K27me1 and H3K27me2, but like

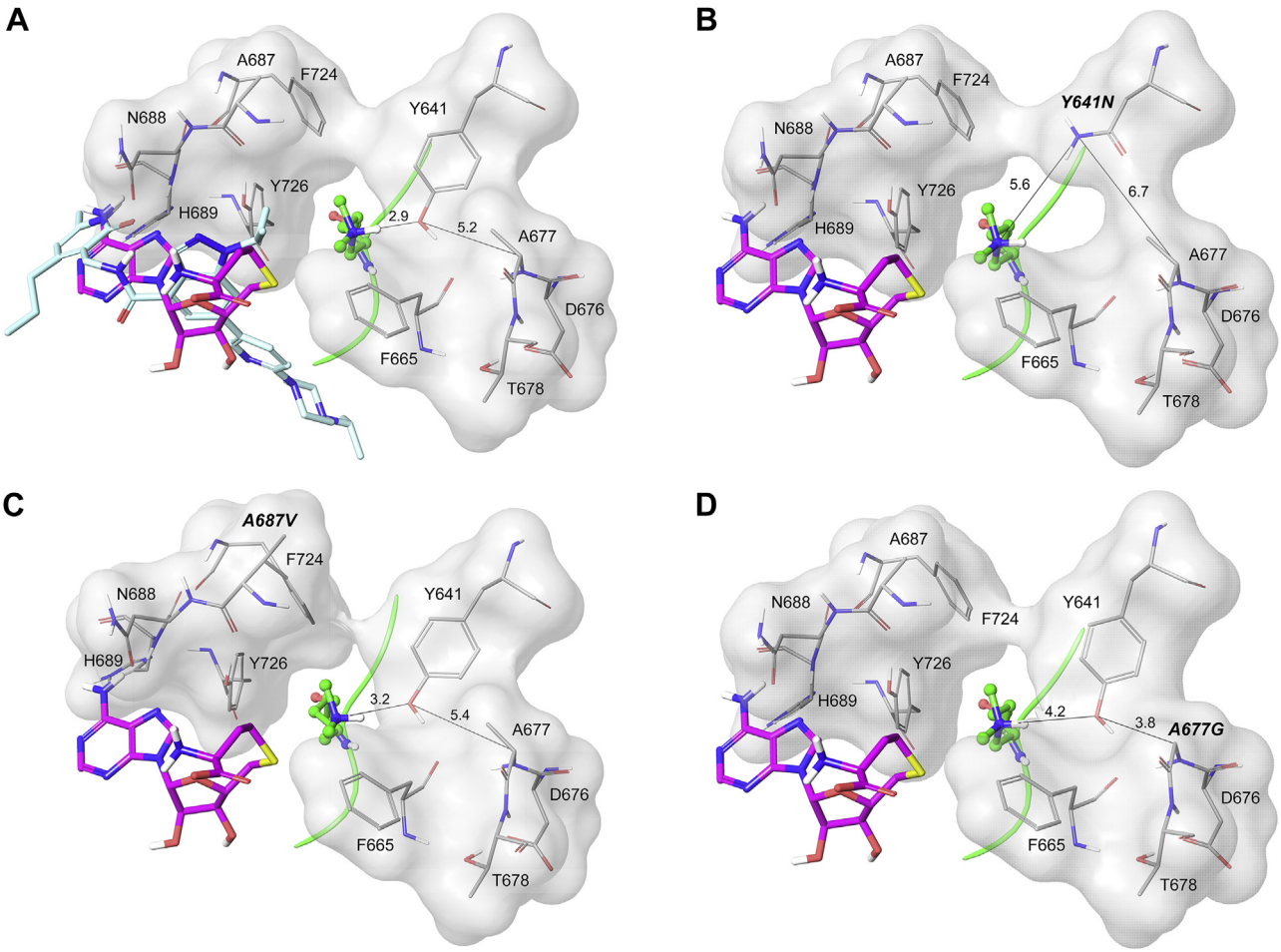

Figure 2. Mechanistic insights into substrate selectivity of EZH2 mutants. (**A**) Wild-type EZH2 uses Y641 to control the methylation state of H3K27 by maintaining a hydrogen bond (*dotted line*, 2.9 Å) with the dimethylated substrate (*green*). SAH (*purple*), the methylation product of cofactor SAM, and the SAM-competitive EZH2 inhibitor UNC1999 (*light blue*) are also included in the model to illustrate their overlapped binding sites. (**B**) Mutation to Y641N enlarges the pocket and reduces the propensity for a hydrogen bond between the asparagine and H3K27me2 (*dotted line*, 5.6 Å), promoting trimethylation of H3K27. (**C**) Mutation of A677 to the smaller glycine residue (A677G) leads to a slight conformational change, enlarging the pocket and weakening the hydrogen bond between Y641 and H3K27me2 (*dotted line*, 4.2 Å), which enhances the ability of $EZH2^{A677G}$ to trimethylate H3K27. (**D**) Mutation of A687 to valine (A687V) leads to a similar conformational change, which leads to a slightly larger pocket, and reduces the strength of the hydrogen bond between Y641 and H3K27me2 (*dotted line*, 3.2 Å). *SAM* = *S*-adenosylmethionine.

$PRC2\text{-}EZH2^{Y641N}$, is incapable of methylating H3K27me0 [31,32,36,37] (Fig. 1B, *purple bars*). Collectively, lymphoma-associated mutations of *EZH2* are gain-of-function mutations and induce PRC2 hyperactivity through distinct molecular mechanisms, leading to a globally elevated H3K27me3 phenotype seen in patient-derived lymphoma cells [26]. The reduced ability to methylate H3K27me0 by EZH2 bearing the hotspot mutation provides an explanation for the exclusively heterozygous mutation pattern observed in lymphoma patients [22,31,32]. A potentially additional aspect of this "cooperative" model is existence of EZH1 in vivo, to which we believe the model can be extended. In the future, generation of a murine *EZH2* Y641-mutated knockin model that faithfully recapitulates the human disease would be very useful for proving the "cooperative" model genetically.

Homology modeling [31,34] and the recently solved apo structure of the EZH2 SET domain [38,39] have provided mechanistic insights into the aforementioned trends in substrate specificity that was seen with the EZH2 gain-of-function mutants. Y641 is believed to be important for both recognizing and limiting the H3K27 methylation states. Specifically, the ε-amino lysine nitrogen of the H3K27 substrate is within proximity of the phenolic oxygen of Y641 to create a hydrogen bond (Fig. 2A, *dashed line*, 2.9 Å) [31,34]. This hydrogen bond is maintained through successive steps of mono- and dimethylation and is believed to be part of the reason $EZH2^{WT}$ prefers to stop methylation with an H3K27me2 product [31]. The functional relevance of this hydrogen bond is manifold [31]: first, it is important for engaging H3K27me0 as substrate, because all Y641 mutants possess little catalytic

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activity with unmethylated substrates (Fig. 1B, H3K27me0); second, Y641 helps create a constrained "pocket" that reduces the ability of H3K27me2 to rotate and accept a third methyl group from the methyl donor (Fig. 2A). A mutation that results in a smaller amino acid at the Y641 position, such as $EZH2^{Y641N}$ (Fig. 2B), changes both the hydrogen bonding ability and local geometry of the substrate-binding pocket. $EZH2^{Y641N}$ is not as proficient at hydrogen bonding with H3K27, because the mutated N641 form is no longer within the acceptable range to form a hydrogen bond [31] (Fig. 2B, *dashed line*, 5.6 Å). Furthermore, as the pocket dimensions change, the dimethylated lysine of the H3K27me2 substrate is able to rotate more freely, which accelerates the transfer of an additional methyl group. Similar effects were observed for other EZH2 Y641 mutations (Y641 to F, C, S, or H), but the most drastic effects were seen with $EZH2^{Y641N}$ [31].

Structural modeling also provided an explanation for the altered substrate preference associated with the EZH2 A677G and A687V mutations. A677 and A687 are two residues that surround the substrate recognition groove and appear to play no direct role in substrate recognition (Fig. 2C, D); however, their mutation leads to a change in geometry of the substrate binding groove, which alters the kinetics of the corresponding PRC2 complex. This change in geometry has two effects on substrate recognition [31,37]: first, both mutations result in a slightly larger pocket, which allows for more rotation of the lysine residue; second, the phenolic oxygen of Y641 is shifted further away from H3K27, resulting in a less stable hydrogen bond, which reduces the ability of EZH2 to recognize the methylation state (Fig. 2C and D, 3.2 Å and 4.2 Å). Because of the smaller effect on pocket dimensions seen in the A677G and A687V mutants, they are less effective in generating H3K27me3 than Y641N mutants (Fig. 1B, H3K27me2 bars) [31], but $EZH2^{A677G}$ is as effective as $EZH2^{WT}$ at methylating H3K27me0 (Fig. 1B, *green bars*). A recent electron microscopy analysis of PRC2 complexes has indicated that the EZH2 SET domain can form intracomplex interactions with other PRC2 components [40]. Therefore, further investigations aimed at solving the structural details of PRC2 complexes will allow a better understanding of PRC2/EZH2 enzymology and its lymphoma-associated mutations.

Development of PRC2 inhibitors

As EZH2 overexpression is frequently found in various human cancers [3], EZH2 and PRC2 are proposed as attractive drug targets for cancer therapy, which has inspired several pharmaceutical companies to endeavor on high-throughput screening campaigns for inhibitors of EZH2. Independent efforts by Epizyme, GlaxoSmithKline (GSK), and Novartis have revealed a structurally similar, cofactor-competitive, small molecule, which potently suppresses EZH2-catalyzed H3K27me3 [41–47] (Table 1). These independent hits were then optimized via traditional medicinal chemistry to enhance the drug-likeness of these compounds.

The first two published EZH2 inhibitors are EPZ005687 [42] and GSK126 [41] (Table 1), which potently inhibit wild-type and lymphoma-associated mutants of EZH2 and are highly selective for EZH2 over a range of unrelated methyltransferases. Although EPZ005687 from Epizyme was not suitable for *in vivo* studies, it was an essential tool compound for target validation and overall study of EZH2 biology. GSK126 from GSK exhibited *in vivo* potency via intraperitoneal administration [41]. These compounds share very similar pharmacophoric features and are fairly selective for EZH2 versus EZH1, the only EZH2-related enzyme (50- to 150-fold selectivity [Table 1]), indicating the high specificity of these compounds. Soon after the disclosure of GSK126, GSK343 was published with similar potency against EZH2, and differs from GSK126 in that it contains an indazole core and several different substitutions such as the piperazine-substituted pyridine (Table 1). El1 (Table 1) is a compound that was optimized from a hit of a high-throughput screening campaign at Novartis [43] and has structural features and selectivity similar to those of EPZ005687 and GSK126, but did not have any *in vivo* activity. UNC1999 (Table 1) represents the first orally bioavailable inhibitor of EZH2 and is the most panactive EZH2/1 inhibitor to date [44]. UNC1999 is similar in structure to GSK343, differing only in the substitution of the pyridine and the capping group of the piperazine. However, this small modification has a large effect on the pharmacokinetic properties of the compound and EZH2/1 selectivity. EZH1 compensates the function of EZH2 [48,49] and emerges as a regulator of hematopoietic neoplasms [50,51]. This EZH2/1 panactivity poses a potential advantage for UNC1999 in cancer cells that rely on both EZH enzymes such as *MLL*-rearranged acute leukemia. Epizyme also released an orally bioavailable derivative of EPZ005687—EPZ-6438 [45] (Table 1). Unlike previously published compounds that contain a bicyclic core, EPZ-6438 has a phenyl core. The only non–pyridone-containing inhibitor of EZH2 is Constellation Pharmaceuticals compound 3 (Table 1) [46]. This compound is not suitable for *in vivo* studies and has about tenfold selectivity for EZH2 versus EZH1. All of these inhibitors exhibit high potency and high selectivity toward wild-type EZH2 and its lymphoma-associated mutant forms [41–47] (Table 1). In addition, enzymology assays revealed that all of the EZH2/1 inhibitors block EZH2 enzymatic activity through a cofactor *S*-adenosylmethionine (SAM)-competitive mechanism (Fig. 2A; inhibitor in *purple* overlapped with SAM shown in *cyan*), rather than disruption of PRC2 complex formation or alteration of PRC2 protein stability.

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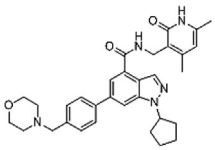
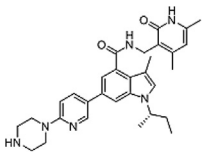
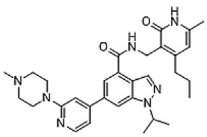
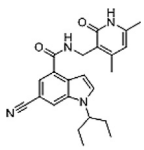
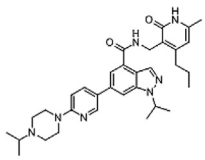
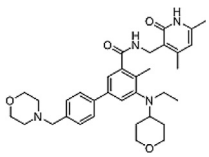
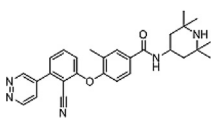
Table 1. Structures of the disclosed small-molecule inhibitors of EZH2, as well as their activity against EZH2, either wild-type (WT) or mutant, and EZH1[a]

Compound	Structure	*In vitro* activity, IC_{50} (nmol/L)[b]					*In vivo* activity?	Orally bioavailable?	Fold selectivity for EZH2
		WT	Y641N	A677G	A687V	EZH1			
EPZ005687		54	41	10	ND	2713	No	No	50
GSK126		10	29	24	441	680	Yes	No	150
GSK343		4	ND	ND	ND	240	No	No	60
El1		9	ND	ND	ND	1340	No	No	140
UNC1999		10	46	ND	ND	45	Yes	Yes	5
EPZ6438		11	38	2	2	392	Yes	Yes	35
Constellation Compound 3		21	197	ND	ND	213	No	No	10

ND = not determined.

[a]Compounds appear in the order they were disclosed in the literature. All compounds except Constellation compound 3 bear a dimethylpyridone motif that is requisite for activity. Additionally, all compounds remain active against EZH2 gain-of-function mutants tested and are fairly selective for EZH2 over EZH1, with UNC1999 displaying the most EZH1 inhibition. GSK126 exhibited the first activity *in vivo*, and UNC1999 was the first EZH2/1 inhibitor to exhibit oral bioavailability.

[b]IC_{50} values were calculated on the basis of the Cheng–Prusoff equation for competitive inhibitors.

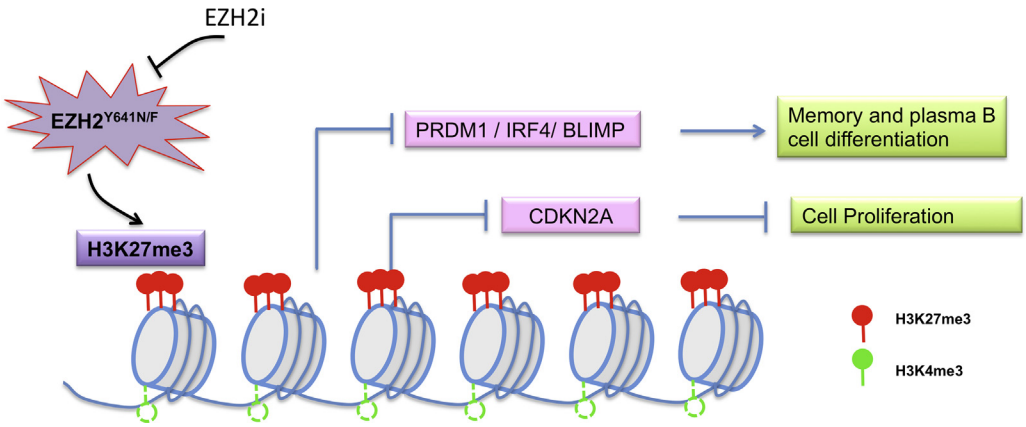


Figure 3. EZH2 gain-of-function mutations in driving B-cell lymphomagenesis. H3K27me3 and H3K4me3 often coexist at multiple genomic loci in germinal-center B cells, rendering these genes in a repressive but poised status. EZH2 gain-of-function mutants reinforce H3K27me3 occupancy and repression of the "bivalent genes" that are crucial for antiproliferation (such as *CDKN2A*) or terminal differentiation (such as PRDM1, IRF4, and BLIMP), promoting hyperplasia or cancerous transformation of germinal-center B cells.

Targeting EZH2 hyperactivity for B-cell lymphoma therapy

Recent studies have indicated that acquisition of EZH2 gain-of-function mutations indeed perturbs normal B-cell development and promotes lymphomagenesis in animal models [14,15,53,54]. Mechanistically, EZH2 gain-of-function mutations cause increased H3K27me3 occupancy and transcriptional repression of critical genes associated with B-cell differentiation, such as *BLIMP1*, *IRF4*, and *PRDM1*, as well as the cell cycle regulator genes *CDKN2A* and *CDKN1A/1B* [14,15,53,54] (Fig. 3). Thus, lymphoma-associated EZH2 mutations are supposed to reinforce B cells at an immature and proliferative state and represent ideal molecular targets. Indeed, early success has been achieved using highly selective EZH2 inhibitors for treatment of GC-cell lymphomas bearing EZH2 gain-of-function mutations [15,41–47]. Studies with GSK126 [41], EPZ005687, and EPZ-6438 [42,55] indicate their preferential effectiveness in suppressing growth of *EZH2*-mutated lymphomas in comparison to those with wild-type EZH2. However, B-cell studies performed in DLBCLs [15], MCLs, and Burkitt's lymphoma [47,56] indicate that overexpression of *EZH2* may confer a similar PRC2 addiction, arguing a general sensitivity to EZH2 inhibitors regardless of EZH2 mutations. Besides cell-based studies, early success with EZH2-selective inhibitors *in vivo* was also achieved in treatment of xenografted DLBCL models in which GSK126 was well tolerated [41]. It remains to be evaluated by several ongoing clinical studies whether the selective PRC2 inhibitors can provide clinical benefits to lymphoma patients. It is, however, noteworthy that the first two patients reported as responders in a recent Epizyme clinical trial are actually EZH2 wild type, with a mediastinal B-cell lymphoma patient reportedly bearing no germinal center features [57]. These unexpected findings raise questions regarding what genetic markers in these responder cases render sensitivity to EZH2 inhibitors clinically or which of the molecular response predictors identified in preclinical mouse and cell line studies will actually translate to the clinic.

Collectively, PRC2 inhibition represents a promising way to treat B-cell lymphomas, especially those DLBCLs, FLs, and MCLs that exhibit PRC2 dependency because of *EZH2* somatic mutation or overexpression.

Crosstalk between PRC2 and other chromatin regulatory pathways

Evidence from different cellular and organismal systems has revealed complex crosstalk between PRC2 and other chromatin machineries. PRC2 activity is influenced by a myriad of cooperative and antagonistic interactions, allowing dynamic regulation of PRC2 target genes and the landscape of H3K27me3. It can be anticipated that certain genetic events during cancer development may affect these crosstalk and genetic interactions, thus conferring a "synthetic" or collateral sensitivity to PRC2 or EZH2 blockade.

Several mechanisms were found to reverse or interfere with PRC2 enzymatic activity (Fig. 4). While PRC2 establishes or "writes" H3K27me3 on chromatin, the KDM6 family of H3K27 demethylases such as UTX removes or "erases" the mark by converting H3K27me3 to the low- or unmethylated H3K27 [58]. Furthermore, catalysis of H3K27me3 is influenced by the surrounding chromatin environment, such as the existence of other histone modifications [59,60] and nucleosomal density [61]. Methylation of histone H3 Lys 4 (H3K4me) and methylation of histone H3 Lys 36 (H3K36me) are two prominent histone marks that demarcate the promoter and gene body regions, respectively, of actively transcribed genes [3,62]. Pre-existence of H3K4me3 or H3K36me2/3 on nucleosomes directly inhibits enzymatic activities of PRC2 [59,60]. In addition,

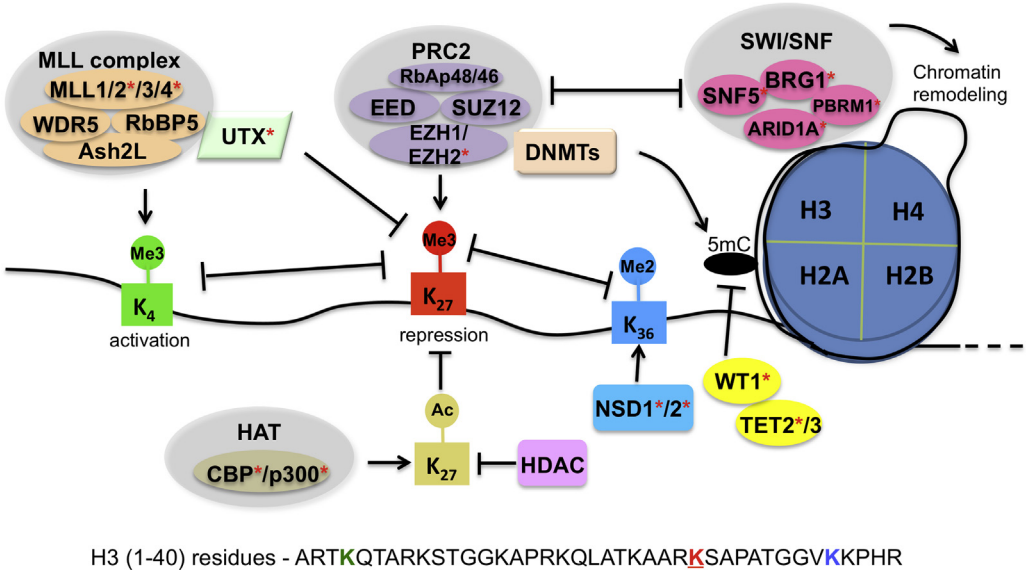


Figure 4. Crosstalk between PRC2 and other epigenetic factors. PRC2 is the sole methyltransferase complex capable of catalyzing H3K27me3 to induce and enforce gene repression. Various epigenetic machineries have crosstalk with PRC2 complex, either cooperatively or antagonistically. The KDM6 family of lysine demethylases such as UTX removes H3K27me3. Methylations of H3K4 and H3K36, as well as acetylation of H3K27, are prominent histone marks associated with transcriptional activation, which are established by the MLL complexes, the NSD family proteins, and the CBP/p300 acetyltransferases, respectively; preinstallation of H3K4me3, H3K36me2/3, and/or H3K27ac inhibits the activity of PRC2 and interferes with H3K27me3 installation. UTX physically interacts with the MLL complex linking H3K4 methylation with H3K27 demethylation. SWI/SNF is an ATP-dependent chromatin remodeling complex that can also antagonize PRC2. PRC2 was found to interact with other epigenetic factors such as histone deacetylases (HDACs) and DNA methyltransferases (DNMTs) to further reinforce a repressive state of polycomb target genes. WT1, however, serves as a platform for recruiting DNA demethylases TET2 and TET3, thus facilitating conversion of 5-methylcytosine (5 mC) to 5-hydroxymethylcytosine (5hmC) and attenuating the DNA methylation-mediated repression. Genetic alterations that affect such a myriad of epigenetic factors (*red stars*) are identified as the recurrent event in various hematopoietic malignancies, causing global or focal perturbation of PRC2 activity and H3K27me3 and thereby rendering cancer cells sensitive to PRC2 inhibition.

because a single lysine cannot be simultaneously modified by methylation and acetylation at the ε-amino group, acetylation of H3K27 (H3K27ac) by histone acetyltransferases such as p300 and CBP also strongly suppresses addition of H3K27me3 by PRC2 [63,64]. Thus, the existence of active histone marks (H3K4me3, H3K36me3/2, H3K27ac) and associated enzymatic machineries directly inhibits PRC2-mediated H3K27me3 establishment (Fig. 4).

Furthermore, genetic studies in *Drosophila* have revealed that the SWI/SNF family of chromatin remodeling complexes antagonizes PRC2 (Fig. 4): mutation of *SWI/SNF* suppressed developmental defects caused by mutation of PRC2 homologues in flies [65]. Although the exact underlying mechanism remains unclear, SWI/SNF remodeling complexes modify DNA accessibility and nucleosomal density [66], a factor known to influence PRC2 enzymatic activity [61]. It has been hypothesized that interplay between SWI/SNF and PRC2 complexes defines a dynamic balance of the chromatin state [67].

Factors also exist that cooperate with PRC2 and promote its function (Fig. 4). For instance, PRC2 physically interacts with the histone deacetylases HDAC1-3 [68], which remove the acetyl group from H3K27ac and thus make the lysine available for methylation by PRC2. PRC2 also associates with *de novo* DNA methyltransferases [69], and it was reported that H3K27me3 and PRC2 targets are positively correlated to DNA methylation [11,69–71].

Taken together, mounting data have indicated that PRC2 activity and functional outcomes are regulated by additional chromatin contexts. Epigenetic factors that influence PRC2 activity or catalytic outcomes such as UTX [72,73] and MMSET/NSD2 [74,75] were recurrently found mutated in a range of hematopoietic malignancies. We therefore anticipate that these genetic lesions may potentially affect PRC2 and confer tumor cell sensitivity to PRC2 inhibition. In the next sections, we summarize recent studies that have indicated PRC2 inhibition as a promising therapeutic option in several genetically defined hematologic malignancies. These studies have expanded the potential applications of PRC2 inhibitors and will improve the personalized medicine and treatment of hematologic cancers in future.

Genetic lesions that confer PRC2 dependence and sensitivity to PRC2 inhibition

UTX mutation

UTX (also known as KDM6A) is a ubiquitously expressed H3K27 demethylase that antagonizes PRC2 in gene regulation and development [76–78]. Localized on the X

chromosome, *UTX* is one of a limited number of genes known to escape X inactivation in females [79]. Somatic mutations of *UTX* were frequently identified in a range of tumors including hematologic cancers such as multiple myeloma (MM), acute lymphoblastic leukemia (ALL), and myeloid leukemia [72,73,80–83] (Fig. 5A). These *UTX* mutations are often loss-of-function gene deletions, truncations and frameshifts, or missense mutations found centered in a hotspot region within the demethylase enzymatic domain [72,80,81]. *UTX* mutations coexist with the hyperactivation mutation of *NOTCH1*, one of the most common lesions associated with T-cell ALLs (T-ALLs) [80]. Deletion of *UTX* significantly accelerated tumor progression in a *NOTCH1* mutation-induced T-ALL model [80], whereas restoration of *UTX* expression in *UTX*-mutated T-ALL cell lines induced apoptosis and suppressed tumor growth [81]. These observations support a *bona fide* tumor-suppressive role for UTX. Although *UTX* deficiency did not change the global levels of H3K27me3 or H3K4me3, it caused a genomewide redistribution of H3K27me3 [72,80,81]. In the *NOTCH1* mutation-induced T-ALL model, loss of *Utx* aberrantly repressed several putative tumor suppressors such as *Pcgf2* and *Lzts2*, which is concurrent with gain in H3K27me3 at their promoters [80] (Fig. 5A). Conversely, gene derepression after *UTX* restoration in *UTX*-null tumor lines was associated with a decrease in H3K27me3 occupancy at target genes [72,80], demonstrating direct involvement of UTX-mediated demethylation in dynamic control of critical gene expression and malignant transformation. Considering the aberrant H3K27me3 accumulation and accompanying gene repression at critical location upon *UTX* mutation, it is reasonable to postulate that *UTX*-mutated leukemia and lymphoma would be more sensitive to PRC2 inhibition. Indeed, van der Meulen et al. have recently reported that *UTX*-mutated T-ALL lines are more sensitive to DZNep, a nonspecific methyltransferase inhibitor that causes EZH2 degradation, than *UTX* wild-type T-ALL lines, despite the similar reduction in H3K27me3 seen in tested lines after compound treatment [80]. Similarly, Ezponda et al. recently reported

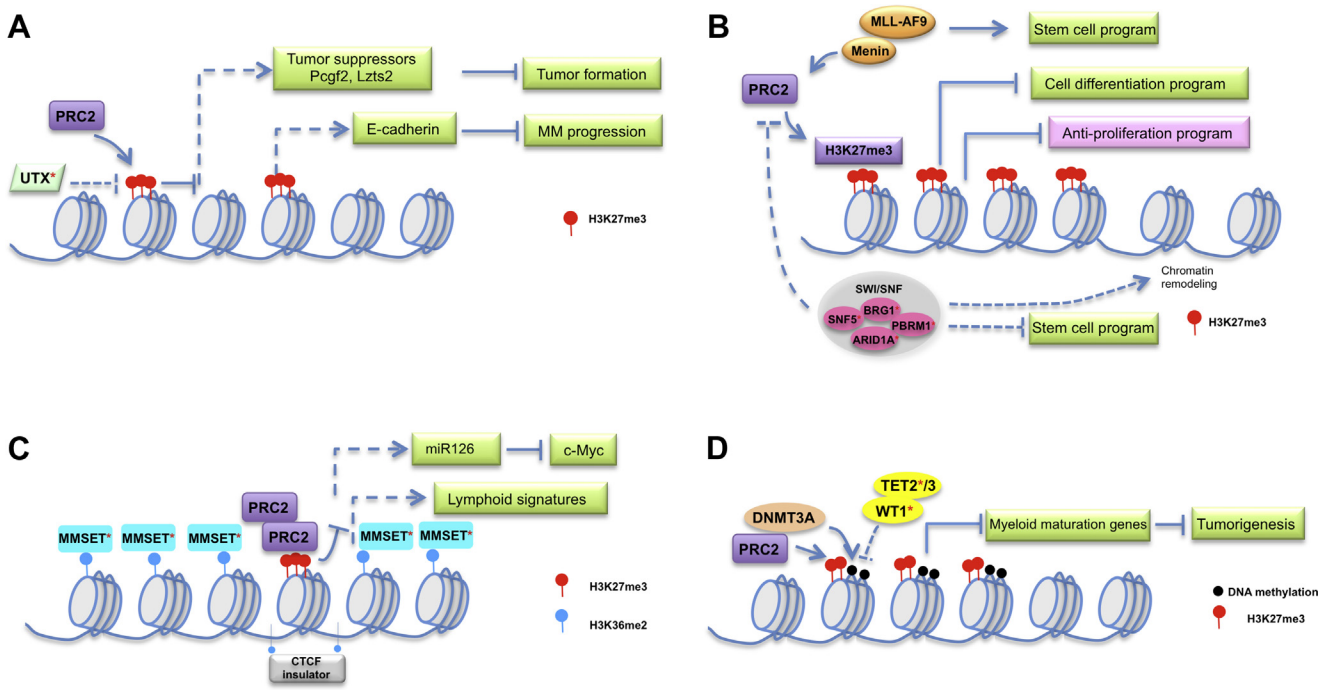


Figure 5. Genetic mutations found in hematopoietic malignancies confer on cancer cells sensitivity to PRC2 or EZH2 inhibition. (**A**) Although they do not affect the global H3K27me3 level, *UTX* inactivating mutations cause focal H3K27me3 enrichment in genes involved in antiproliferation and cell adhesion to promote cancerous transformation. *denotes mutation of the labeled gene found in hematological cancers. (**B**) Biallelic inactivation of a core component of the SWI/SNF chromatin remodeling complex, *SNF5* (also known as *SMARCB1*), enhances PRC2-mediated suppression of antiproliferation and lineage differentiation signature genes; other subunits of the SWI/SNF complex also function as tumor suppressors and their mutations in malignancies may confer a similar PRC2 dependence. Similarly, PRC2 is also required for acute leukemia caused by *MLL* rearrangements such as MLL-AF9. In addition to leukemia stem cell programs, MLL-AF9 oncoproteins directly promote expression of *EZH2*. PRC2 complexes assembled by both EZH2 and EZH1 repress myeloid differentiation and antiproliferation programs to promote acute leukemogenicity. (**C**) Because of the abnormal chromosomal translocation or gain-of-function somatic mutation (E1099K) found in multiple myeloma patients, hyperactivation of MMSET/NSD2 causes a global increase in H3K36me2 and decrease in H3K27me3. However, H3K27me3 is maintained and even enriched at certain critical regions protected by CTCF, an insulator factor. Focal enrichment of EZH2 leads to an enhanced suppression of lymphoid signatures and miR126, a negative regulator of c-*MYC*, thus enhancing tumorigenesis. (**D**) Loss-of-function mutation of *WT1* induces a DNA hypermethylation phenotype in patients with acute myeloid leukemia (AML). Methylated genes found specific to *WT1*-mutated AMLs largely overlap with PRC2 gene targets such as myeloid maturation signatures, indicating cooperation between DNMTs and PRC2. *WT1*-mutated AMLs exhibit sensitivity to PRC2 inhibition.

that GSK343 (Table 1) is more effective in treating *UTX*-mutant MM lines in comparison to the *UTX*–wild-type lines [84]. These findings provide rationale for treating *UTX*-mutated hematopoietic tumors with the more specific PRC2 inhibitors.

Loss-of-function mutation of the SWI/SNF *complex*

The mammalian SWI/SNF complex is an ATP-dependent chromatin remodeling complex that is critical for regulating cell differentiation and proliferation [85]. Loss-of-function mutations that "hit" various components of the SWI/SNF complex have been reported in nearly 20% of human cancers, including hematologic cancers, indicating its tumor suppressive role [86,87] (Figs. 4 [*stars*] and 5B). SNF5 is the first subunit of SWI/SNF functionally linked to PRC2 deregulation during tumorigenesis [88]. Biallelic inactivation of *SNF5* exists in a majority of human malignant rhabdoid tumors (MRTs) [89,90] and sporadic cases of T-cell malignancies [91]. Conditional inactivation of *Snf5* in mouse peripheral T cells causes completely penetrant CD8+ T-cell lymphomas [88]. It has been found that *Snf5* mutation drives tumor progression by disrupting several key pathways related to tumor suppression, differentiation, and cell cycle progression [66]. The mechanisms that underlie functional antagonisms between SNF5 and PRC2 are manifold: first, SNF5 directly downregulates *EZH2* gene expression in tumor cells [88]; in addition, the SWI/SNF complex restricts the recruitment and/or spreading of PRC2 at its critical target genes such as the tumor-suppressive locus $p16^{INK4a}$ and numerous lineage differentiation genes [67,88]. PRC2-mediated repression of $p16^{INK4a}$ is counteracted by SNF5, and occupancy of H3K27me3 was elevated at $p16^{INK4a}$ in *SNF5*-deficient lymphomas [67,88]; restoration of SNF5 in MRT cells caused eviction of polycomb proteins and loss of H3K27me3 at the $p16^{INK4a}$ gene, as well as concomitant increased occupancy of transactivators, leading to its elevated transcription [67] (Fig. 5B). A similar phenomenon was seen at differentiation genes in these *SNF5*-deficient lymphomas [88]. Therefore, in tumors carrying *SNF5* deficiency such as T-cell lymphomas, PRC2 complexes abnormally enforce repression of genes critical for tumor suppression and cell differentiation, keeping cancer cells in a proliferative, undifferentiated state. Indeed, knockdown or deletion of *EZH2* in the *SNF5*-deficient cells reversed $p16^{INK4a}$ repression [88]. These findings indicate PRC2 dependence in *SNF5*-deficient cancers. A recent study found that blockade of PRC2 activity by an EZH2 inhibitor specifically suppressed growth of *SNF5*-deleted and not wild-type MRTs, by inducing apoptosis and cell differentiation in the mutant MRTs [45]. As mutations of other SWI/SNF complex components such as BRG1, ARID1A, and PBRM1 (Figs. 4 and 5B, *stars*) were found in various cancers and are generally less well understood, it would be intriguing to examine if these mutations also confer SWI/SNF loss-of-function and, thus, PRC2/EZH2 dependence. Indeed, two recent studies have reported that *BRG1*-mutated non–small-cell lung cancer (NSCLC) cells [92] and *ARID1A*-mutated ovarian cancer cells [93] exhibit sensitivity to GSK126 (Table 1), an EZH2-selective inhibitor, in comparison to cancer cells without such a lesion. Hematopoietic cancers bearing the similar mutations might be equally sensitive to PRC2 inhibition.

MLL gene rearrangement

The mammalian *mixed lineage leukemia* (*MLL*) gene encodes an H3K4-specific methyltransferase [3,94]. Rearrangement of *MLL* is responsible for ~70% of infant acute myeloid, lymphoid, or mixed-lineage leukemias and ~7%–10% of adult cases [94]. Acute leukemia with *MLL* rearrangement has a poor prognosis with low survival rates, highlighting the need for novel molecularly targeted interventions [95,96]. It has been found that MLL fusion oncoproteins produced by *MLL* gene rearrangements recruit epigenetic factors and/or transcriptional elongation-promoting complexes such as DOT1L and bromodomain proteins (BRD4) to enforce abnormal activation of oncogenes such as *HOX*, *MEIS1*, and *c-MYC* [3,94–96]. In addition, studies have also found that MLL fusion oncoproteins are recruited to the promoter of *EZH2* and promote its transcription [97] (Fig. 5B), indicating a role for PRC2 in *MLL*-rearranged leukemia. Indeed, several works have found that PRC2 acts in parallel with *MLL* rearrangements by controlling a distinctive program to sustain leukemogenicity [50,51,98]. In detail, PRC2 directly represses genes that are critical for myeloid differentiation such as *EGR1*, as well as genes that limit proliferation and self-renewal of hematopoietic cells such as *CDKN2A* [50,51,98]. In addition, EZH2 was also found to physically interact with C/EBPα, a differentiation-promoting transcription factor, and repressed the C/EBPα-mediated prodifferentiation program [97]. In the *MLL*-rearranged acute leukemia, both *EZH2* and *EZH1* are expressed and compensate one another to promote acute leukemogenesis [50,51]. Disruption of both enzymes is required to inhibit growth of leukemia carrying *MLL-AF9*, a common form of *MLL* rearrangements [50,51]. UNC1999, the most panactive inhibitor of both EZH1 and EZH2 to date (Table 1), indeed demonstrated a unique growth-suppressing effect on a panel of *MLL*-rearranged leukemia cells *in vitro* by inhibiting their repopulating ability and promoting cell differentiation and apoptosis, whereas GSK126, the EZH2-selective inhibitor with much less activity against EZH1 (Table 1), failed to efficiently inhibit H3K27me3 or suppress proliferation of *MLL*-rearranged leukemia cells [52]. Genomic profiling by ChIP-seq revealed the molecular events following PRC2 enzymatic inhibition: UNC1999 preferentially "erases" H3K27me3 associated with distal regulatory

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elements such as enhancers and remodels the landscape of H3K27me3 versus H3K27ac at proximal promoters, leading to derepression of numerous PRC2 target genes that include *CDKN2A* and development/differentiation-related genes [52]. Oral administration of UNC1999 prolonged survival of a well-defined murine leukemia model bearing *MLL-AF9*, a common form of *MLL* rearrangement [52]. Therefore, the targeting of both PRC2-EZH2 and PRC2-EZH1 with small-molecule compounds such as UNC1999 represents a new way of treating *MLL*-rearranged leukemia; a similar tumor-suppressing phenomenon was also observed in *MLL*-rearranged leukemia using a hydrocarbon-stapled peptide recently developed to disrupt physical interactions of EZH2 and EZH1 with its cofactor EED, which leads to the proteasome-mediated degradation of PRC2 complexes [99].

Translocation and activating mutation of MMSET/NSD2

MMSET (also known as NSD2 or WHSC1) is a histone methyltransferase that catalyzes dimethylation of histone H3 at lysine 36 (H3K36me2), a histone modification associated with transcriptional initiation and/or elongation [100]. Reciprocal t(4;14) translocation, which results in *MMSET* fusion to the immunoglobulin heavy chain locus and, hence, overexpression of *MMSET*, was reported in 15%–20% of multiple myeloma patients [101]. This genetic event is believed to drive tumorigenesis of myeloma and is associated with lower survival rates of myeloma patients [102–105]. Consistently, a recurrent gain-of-function mutation of *MMSET* (E1099 K) was also identified in a subset of ALL patients [74,75] and specifically targets the catalytic SET domain of MMSET. In both cases, the activated MMSET reprograms the landscape of histone modifications, inducing a global increase in H3K36me2 and concurrent decrease in H3K27me3, caused by the antagonistic effect of H3K36me2 on PRC2 and, thus, H3K27 methylation (Figs. 4 and 5C) [74,106,107]. However, despite a genomewide net loss of H3K27me3, this silencing mark is maintained and even enriched further at certain specific loci [106]. Motif analysis suggested that CTCF, a known genome organizer and insulator, is likely to be responsible for preventing these PRC2/H3K27me3 domains from the intrusion of MMSET [106]. This study indicates that although the hyperactivated MMSET restricts the binding and activity of PRC2 at H3K36me2-demarcated loci, it also induces a collateral effect at putative "CTCF-protected" loci because the free pool of PRC2 has nowhere else to bind [106]. As a result, the latter loci end up with enhanced PRC2 binding and a concomitant elevated level of H3K27me3, leading to abnormal repression of the embedded genes [106] (Fig. 5C). Further genomic profiling revealed that genes embedded within the retained PRC2 domains include the GC B cell-associated gene signatures, as well as critical microRNAs such as miR-126, a negative regulator of *c-MYC* [106] (Fig. 5C), suggesting a critical role for certain newly acquired PRC2-binding sites in promotion of growth and aggressiveness of myeloma cells with MMSET hyperactivation. Indeed, myeloma cells carrying t(4;14) *MMSET* translocations exhibit more sensitivity to GSK343 (Table 1) than t(4;14)-negative myeloma cells [106]. GSK343 treatment also led to elevated miR-126 levels and concomitant decreased MYC levels. Therefore, although additional studies need to be carried out, PRC2 inhibitors represent a promising therapeutic agent for treatment of myeloma patients with MMSET hyperactivity.

Inactivating mutations of WT1

The *Wilms' tumor 1* (*WT1*) gene encodes a sequence-specific zinc finger transcription factor that was originally identified as a tumor suppressor in Wilms' tumor, a rare kidney cancer [108]. WT1 has been linked to regulation of critical differentiation genes in various biological processes, particularly nephrogenesis and hematopoiesis [109]. Heterozygous somatic mutations of *WT1* were identified in approximately 10% of cases of acute myeloid leukemia (AML), with the hotspot mutations centered within the DNA-binding zinc finger domains [110,111]. These loss-of-function mutations of *WT1* correlate with poor prognosis and chemotherapy resistance in AML patients, but the role of WT1 and oncogenic mechanisms that underlie *WT1*-mutated AMLs is unclear [111]. Recent studies have linked *WT1* mutations to regulation of DNA methylation [112–114] (Figs. 4 and 5D). WT1 physically interacts with the methylcytosine dioxygenases TET2 and TET3, and serves as one of the mechanisms responsible for recruitment of TET2/3 to target loci (Fig. 4), where TET proteins mediate DNA demethylation via conversion of 5-methylcytosine (5 mC) to 5-hydroxymethylcytosine (5hmC) and subsequent oxidative derivatives [112,113]. Inactivated mutants of WT1 in AMLs lack the critical DNA binding domains, leading to a DNA hypermethylation phenotype in the affected AMLs [112–114]. Indeed, introduction of mutant *WT1* into *WT1*–wild-type AML cells is sufficient in inducing DNA hypermethylation, indicating a causal role for *WT1* mutation in regulating DNA methylation [114]. Intriguingly, the DNA hypermethlylated loci were found enriched with signature of known PRC2 targets such as differentiation genes [114], indicating cooperation between DNA hypermethylation and PRC2 (Fig. 5D). Consistently, the mutant and not wild-type WT1 blocked myeloid differentiation of hematopoietic stem cells [114]. Moreover, EZH2 is significantly overexpressed in *WT1*-mutated AMLs compared with *WT1*–wild-type cases [114] (Fig. 5D). EZH2 knockdown or its blockade with the inhibitor GSK126 promoted myeloid differentiation in AML cells carrying *WT1* mutations [114]. These studies have provided rationale for using EZH2 inhibitors in the treatment of *WT1*-mutated AMLs.

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Potential adverse, side, and toxic effects of PRC2 or EZH2 inhibition

Despite enthusiasm shared by the community for EZH2/1 inhibitors as a future cancer therapy, recent studies have raised concerns over the potentially side, toxic, or even adverse effects caused by PRC2/EZH2 blockade.

First, in contrast to the oncogenic function of PRC2 and EZH2 described above, inactivating mutation of EZH2 and other PRC2 components has emerged as a recurrent theme in a range of human cancers, which demonstrates a dichotomous role for PRC2 in different biological contexts and raises concerns over PRC2 and EZH2 inhibition in clinical treatment. Specifically, various damaging mutations, such as biallelic deletion and missense, frameshifting, or truncation mutations, that "hit" either the PRC2 core component (EZH2, EED, and SUZ12) [115–117] or cofactor genes (JARID2 [118] and ASXL1 [119]) are recurrent in a range of myeloid neoplasms such as myelodysplastic syndromes (MDSs), primary myelofibrosis (PMF), myeloproliferative neoplasms (MPNs), and chronic myelomonocytic leukemia (CMML); similar PRC2-inactivating somatic mutations also occur frequently among human T-ALL [120–122] and solid tumors such as malignant peripheral nerve sheath tumors (MPNSTs) [123–126]. These PRC2-inactivating mutations are associated with adverse prognostic outcomes of MDS and PMF patients [127]. The tumor-suppressive role of PRC2 has been verified with several murine cancer models: deletion of *Ezh2* alone in hematopoietic systems is sufficient to cause spontaneous T-ALL with an average latency of 150 days in mice [122]; *Ezh2* loss also led to development of an MDS/MPN-like phenotype following serial bone marrow transplantation [128]; loss of PRC2 caused aberrant gene transcription programs and significantly enhanced *in vivo* tumorigenesis driven by mutant RAS signaling [123–126]. These recent findings have collectively indicated that EZH2 and PRC2 act as *bona fide* tumor suppressors in certain cell lineages or biological contexts.

In addition, EZH2/1 or PRC2 was also reported to be important in normal development. In particular, *EZH1* is documented as a crucial factor in maintaining the self-renewal capacity of adult HSCs by protecting them from senescence [13]; similar developmental roles for EZH2 or EZH1 were reported as well in other tissue-specific stem and progenitor cells [129,130]. A dual EZH2 and EZH1 inhibitor may therefore cause greater toxicity by impairing normal functions of the PRC2 assembled by both enzymes.

Collectively, these recent advances have urged the field to re-evaluate the clinical application of PRC2 and EZH2 inhibitors with more caution. A safe therapeutic window needs to be determined to eradicate cancer cells over generation of the unwanted toxic or even adverse effect during treatment. Furthermore, efforts are required to explore the cancer genetic backgrounds that define either beneficial or adverse effects of PRC2/EZH2 inhibition. Indeed, a recent study of NSCLCs indicated that usage of EZH2 inhibitors can cause differential or even opposite effects among NSCLC patients carrying different genetic backgrounds: although EZH2 inhibition sensitizes the *BRG1*-mutated NSCLCs to etoposide, a topoisomerase II inhibitor, and improves cancer treatment, the same EZH2 inhibitor causes an opposite effect by conferring *BRG1* wild-type NSCLC resistance on topoisomerase II inhibitors [92].

Combination therapy and drug resistance

PRC2 inhibitors can be potentially used with other Food and Drug Administration-approved agents, either sequentially or in a combinational therapeutic strategy, to further enhance their therapeutic value. Indeed, pretreatment with EZH2 inhibitor sensitized *BRG1*-mutated NSCLCs to the topoisomerase II inhibitors, and dual treatment with EZH2 and topoisomerase II inhibitors provided synergy in reversing oncogenicity [92]. A similar and dramatic synergistic antitumor effect was observed when combining EZH2 inhibitors with Food and Drug Administration-approved glucocorticoid receptor agonists such as prednisolone and dexamethasone in the treatment of lymphomas, regardless of *EZH2* mutation status [131]. However, the molecular basis for these described synergistic effects remains unclear. Use of a PRC2 inhibitor in combination therapy with other developed agents, for example, inhibitor of histone deacetylase, will also be explored (Fig. 4), because PRC2 and histone deacetylase are known to interact physically in their coordinated action in mediating gene silencing [26].

In addition, it is probably not of a total surprise that tumor cells may develop strategies such as the acquired additional mutations that confer resistance to EZH2/1 inhibitors, because clinically relevant and inhibitor-resistant mutations were found during treatment with inhibitors of oncogenic kinase such as BCR-ABL [132] and B-RAF [133]. Indeed, a very recent cell line–based study identified the two missense mutations of *EZH2* that affect compound binding and therefore confer resistance to several tested EZH2 inhibitors [134]. One of such acquired mutations, *Y661D*, occurs *cis* in the *EZH2 Y641*-mutated allele and is in the vicinity of the SAM pocket where these inhibitors are believed to bind (Fig. 2A); however, the other mutation, *Y111L*, is found in the wild-type *EZH2* allele, thus providing additional support for the model of cooperativity between wild-type and mutant EZH2 enzymes. The fact that Y111L is far from the SAM-binding pocket (located between the EED-binding domain and the SANT domain) suggests a long-range interaction potentially via formation of a high-order PRC2 complex for mediating regulation or formation of an active methyltransferase domain of EZH2. Research is required to learn the structural details of EZH2/1 inhibitors bound to EZH2 or the PRC2 complex, which will enable the rational design of new strategies for inhibiting the inhibitor-resistant mutations that tumors can acquire during treatment.

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Conclusions

Therapeutic targeting of PRC2 initially emerged from studies reporting that overexpression or hyperactive mutation of *EZH2* can initiate, promote, or maintain oncogenesis. The already developed small-molecule inhibitors of PRC2 or EZH2/1 have achieved early success in the laboratory and are currently under clinical evaluation for the treatment of germinal-center B-cell lymphomas bearing *EZH2* mutations, which account for about 15%–20% of all cases. Studies have revealed the genetic interaction and functional crosstalk (either cooperative or antagonistic) of PRC2 with a variety of other chromatin modifiers and epigenetic regulators such as SWI/SNF, UTX, and MMSET. Somatic mutations that affect these epigenetic factors have been identified as a recurrent genetic event in a range of hematopoietic malignancies, which cause a collateral dependence of PRC2 and an increased sensitivity to pharmacologic inhibition of PRC2 or EZH2. In addition, increasing evidence has started to reveal the PRC2-independent roles of EZH2/1 in various cancer and biological contexts [135–137], and further investigations shall be extended to studies of their nonhistone substrates [138] and/or noncanonical gene-activation roles [139,140]. Thus, we expect to see an increasing list of such PRC2 or EZH2 dependencies in the future. For example, the trithorax group proteins, such as the MLL family of H3K4-specific methyltransferases and histone acetyltransferases CBP/p300, are known to antagonize PRC2 (Fig. 4) [3], and inactivating somatic mutations of *MLL2*, *MLL4*, *CBP*, and *p300* are frequent in lymphomas [23,24,141]. In addition to EZH2 gain-of-function mutations, identification of collateral PRC2 or EZH2 dependency in various hematopoietic malignancies has expanded the potential application of the already developed PRC2 inhibitors and will promote the precision medicine and personalized therapy. However, considering the fact that PRC2 or EZH2 has a tumor-suppressive role with loss-of-function mutations recurrently identified in various human cancers, we need to avoid treating cancer patients with certain genetic lesions and backgrounds with EZH2 or PRC2 inhibitors because such blockade could accelerate cancer progression, as reported in recent studies [92,123–126]. Although ongoing efforts continue to optimize the pharmacokinetic and pharmacodynamic properties of the currently existing PRC2 and EZH2/1 inhibitors, as well as development of second-generation inhibitors to target additional mutations of *EZH2* that tumors acquire to produce inhibitor resistance, we remain optimistic and excited about their future clinical applications in hematologic cancer, as well as a broader spectrum of human cancers being unraveled to show PRC2 dependency and, thus, PRC2 inhibitor sensitivity.

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Conflict of interest disclosure

The authors have no conflicting financial interests to disclose.

References

1. Strahl BD, Allis CD. The language of covalent histone modifications. Nature. 2000;403:41–45.
2. Schubeler D. Function and information content of DNA methylation. Nature. 2015;517:321–326.
3. Chi P, Allis CD, Wang GG. Covalent histone modifications—Miswritten, misinterpreted and mis-erased in human cancers. Nat Rev Cancer. 2010;10:457–469.
4. Wang GG, Allis CD. ChiChromatin remodeling and cancer: Part II. ATP-dependent chromatin remodeling. Trends Mol Med. 2007;13:373–380.
5. Weber CM, Henikoff S. Histone variants: Dynamic punctuation in transcription. Genes Dev. 2014;28:672–682.
6. Shih AH, Abdel-Wahab O, Patel JP, Levine RL. The role of mutations in epigenetic regulators in myeloid malignancies. Nat Rev Cancer. 2012;12:599–612.
7. Dawson MA, Kouzarides T. Cancer epigenetics: From mechanism to therapy. Cell. 2012;150:12–27.
8. Rodriguez-Paredes M, Esteller M. Cancer epigenetics reaches mainstream oncology. Nat Med. 2011;17:330–339.
9. Arrowsmith CH, Bountra C, Fish PV, Lee K, Schapira M. Epigenetic protein families: A new frontier for drug discovery. Nat Rev Drug Discov. 2012;11:384–400.
10. Margueron R, Reinberg D. The polycomb complex PRC2 and its mark in life. Nature. 2011;469:343–349.
11. Di Croce L, Helin K. Transcriptional regulation by polycomb group proteins. Nat Struct Mol Biol. 2013;20:1147–1155.
12. Mochizuki-Kashio M, Mishima Y, Miyagi S, et al. Dependency on the polycomb gene Ezh2 distinguishes fetal from adult hematopoietic stem cells. Blood. 2011;118:6553–6561.
13. Hidalgo I, Herrera-Merchan A, Ligos JM, et al. Ezh1 is required for hematopoietic stem cell maintenance and prevents senescence-like cell cycle arrest. Cell Stem Cell. 2012;11:649–662.
14. Caganova M, Carrisi C, Varano G, et al. Germinal center dysregulation by histone methyltransferase EZH2 promotes lymphomagenesis. J Clin Invest. 2013;123:5009–5022.
15. Beguelin W, Popovic R, Teater M, et al. EZH2 is required for germinal center formation and somatic EZH2 mutations promote lymphoid transformation. Cancer Cell. 2013;23:677–692.
16. Musselman CA, Gibson MD, Hartwick EW, et al. Binding of PHF1 Tudor to H3K36me3 enhances nucleosome accessibility. Nat Commun. 2013;4:2969.
17. Cai L, Rothbart SB, Lu R, et al. An H3K36 methylation-engaging Tudor motif of polycomb-like proteins mediates PRC2 complex targeting. Mol Cell. 2013;49:571–582.
18. Ballare C, Lange M, Lapinaite A, et al. Phf19 links methylated Lys36 of histone H3 to regulation of polycomb activity. Nat Struct Mol Biol. 2012;19:1257–1265.

ARTICLE IN PRESS

19. Pasini D, Cloos PA, Walfridsson J, et al. JARID2 regulates binding of the polycomb repressive complex 2 to target genes in ES cells. Nature. 2010;464:306–310.
20. Li G, Margueron R, Ku M, Chambon P, Bernstein BE, Reinberg D. Jarid2 and PRC2, partners in regulating gene expression. Genes Dev. 2010;24:368–380.
21. Brockdorff N. Noncoding RNA and polycomb recruitment. RNA. 2013;19:429–442.
22. Morin RD, Johnson NA, Severson TM, et al. Somatic mutations altering EZH2 (Tyr641) in follicular and diffuse large B-cell lymphomas of germinal-center origin. Nat Genet. 2010;42:181–185.
23. Morin RD, Mendez-Lago M, Mungall AJ, et al. Frequent mutation of histone-modifying genes in non-Hodgkin lymphoma. Nature. 2011; 476:298–303.
24. Okosun J, Bodör C, Wang J, et al. Integrated genomic analysis identifies recurrent mutations and evolution patterns driving the initiation and progression of follicular lymphoma. Nat Genet. 2014;46: 176–181.
25. Van Galen JC, Dukers DF, Giroth C, et al. Distinct expression patterns of polycomb oncoproteins and their binding partners during the germinal center reaction. Eur J Immunol. 2004;34:1870–1881.
26. Wang GG, Konze KD, Tao J. Polycomb genes, miRNA, and their deregulation in B-cell malignancies. Blood. 2015;125:1217–1225.
27. Van Kemenade FJ, Raaphorst FM, Blokzijl T, et al. Coexpression of BMI-1 and EZH2 polycomb-group proteins is associated with cycling cells and degree of malignancy in B-cell non-Hodgkin lymphoma. Blood. 2001;97:3896–3901.
28. Visser HP, Gunster MJ, Kluin-Nelemans HC, et al. The polycomb group protein EZH2 is upregulated in proliferating, cultured human mantle cell lymphoma. Br J Haematol. 2001;112:950–958.
29. Abd Al Kader L, Oka T, Takata K, et al. In aggressive variants of non-Hodgkin lymphomas, Ezh2 is strongly expressed and polycomb repressive complex PRC1.4 dominates over PRC1.2. Virchows Arch. 2013;463:697–711.
30. Van Galen JC, Muris JJF, Oudejans JJ, et al. Expression of the polycomb-group gene BMI1 is related to an unfavourable prognosis in primary nodal DLBCL. J Clin Pathol. 2007;60:167–172.
31. McCabe MT, Graves AP, Ganji G, et al. Mutation of A677 in histone methyltransferase EZH2 in human B-cell lymphoma promotes hypertrimethylation of histone H3 on lysine 27 (H3K27). Proc Natl Acad Sci U S A. 2012;109:2989–2994.
32. Majer CR, Lei J, Scott MP, et al. A687V EZH2 is a gain-of-function mutation found in lymphoma patients. FEBS Lett. 2012;586:3448–3451.
33. Bodör C, Grossmann V, Popov N, et al. EZH2 mutations are frequent and represent an early event in follicular lymphoma. Blood. 2013; 122:3165–3168.
34. Yap DB, Chu J, Berg T, et al. Somatic mutations at EZH2 Y641 act dominantly through a mechanism of selectively altered PRC2 catalytic activity, to increase H3K27 trimethylation. Blood. 2011;117: 2451–2459.
35. Sneeringer CJ, Scott MP, Kuntz KW, et al. Coordinated activities of wild-type plus mutant EZH2 drive tumor-associated hypertrimethylation of lysine 27 on histone H3 (H3K27) in human B-cell lymphomas. Proc Natl Acad Sci U S A. 2010;107:20980–20985.
36. Swalm BM, Knutson SK, Warholic NM, et al. Reaction coupling between wild-type and disease-associated mutant EZH2. ACS Chem Biol. 2014;9:2459–2464.
37. Ott HM, Graves AP, Pappalardi MB, et al. A687V EZH2 is a driver of histone H3 lysine 27 (H3K27) hypertrimethylation. Mol Cancer Ther. 2014;13:3062.
38. Antonysamy S, Condon B, Druzina Z, et al. Structural context of disease-associated mutations and putative mechanism of autoinhibition revealed by X-ray crystallographic analysis of the EZH2-SET domain. PLoS One. 2013;8:e84147.
39. Wu H, Zeng H, Dong A, et al. Structure of the catalytic domain of EZH2 reveals conformational plasticity in cofactor and substrate binding sites and explains oncogenic mutations. PLoS One. 2013;8: e83737.
40. Ciferri C, Lander GC, Maiolica A, Herzog F, Aebersold R, Nogales E. Molecular architecture of human polycomb repressive complex 2. ELife. 2012;1:e00005.
41. McCabe MT, Ott HM, Ganil G, et al. EZH2 inhibition as a therapeutic strategy for lymphoma with EZH2-activating mutations. Nature. 2012;492:108–112.
42. Knutson SK, Wigle TJ, Warholic NM, et al. A selective inhibitor of EZH2 blocks H3K27 methylation and kills mutant lymphoma cells. Nat Chem Biol. 2012;8:890–896.
43. Qi W, Chan HM, Teng L, et al. Selective inhibition of Ezh2 by a small molecule inhibitor blocks tumor cells proliferation. Proc Natl Acad Sci U S A. 2012;109:21360–21365.
44. Konze KD, Ma A, Li FL, et al. An orally bioavailable chemical probe of the lysine methyltransferases EZH2 and EZH1. ACS Chem Biol. 2013;8:1324–1334.
45. Knutson SK, Warholic NM, Wigle TJ, et al. Durable tumor regression in genetically altered malignant rhabdoid tumors by inhibition of methyltransferase EZH2. Proc Natl Acad Sci U S A. 2013;110: 7922–7927.
46. Garapaty-Rao S, Nasveschuk C, Gagnon A, et al. Identification of EZH2 and EZH1 small molecule inhibitors with selective impact on diffuse large B cell lymphoma cell growth. Chem Biol. 2013; 20:1329–1339.
47. Bradley WD, Arora S, Busby J, et al. EZH2 Inhibitor efficacy in non-Hodgkin's lymphoma does not require suppression of H3K27 monomethylation. Chem Biol. 2014;21:1463–1475.
48. Shen X, Liu YC, Hsu YJ, et al. EZH1 mediates methylation on histone H3 lysine 27 and complements EZH2 in maintaining stem cell identity and executing pluripotency. Mol Cell. 2008;32:491–502.
49. Margueron R, Li G, Sarma K, et al. Ezh1 and Ezh2 maintain repressive chromatin through different mechanisms. Mol Cell. 2008;32: 503–518.
50. Neff T, Sinha AU, Kluk MJ, et al. Polycomb repressive complex 2 is required for MLL-AF9 leukemia. Proc Natl Acad Sci U S A. 2012; 109:5028–5033.
51. Shi J, Wang E, Zuber J, et al. The polycomb complex PRC2 supports aberrant self-renewal in a mouse model of MLL-AF9;Nras(G12D) acute myeloid leukemia. Oncogene. 2013;32:930–938.
52. Xu B, On DM, Ma A, et al. Selective inhibition of EZH2 and EZH1 enzymatic activity by a small molecule suppresses MLL-rearranged leukemia. Blood. 2015;125:346–357.
53. Velichutina I, Shaknovich R, Geng H, et al. EZH2-mediated epigenetic silencing in germinal center B cells contributes to proliferation and lymphomagenesis. Blood. 2010;116:5247–5255.
54. Berg T, Thoene S, Yap D, et al. A transgenic mouse model demonstrating the oncogenic role of mutations in the polycomb-group gene EZH2 in lymphomagenesis. Blood. 2014;123:3914–3924.
55. Knutson SK, Kawano S, Minoshima Y, et al. Selective inhibition of EZH2 by EPZ-6438 leads to potent antitumor activity in EZH2 mutant non-Hodgkin lymphoma. Mol Cancer Ther. 2014;13:842–854.
56. Zhang X, Zhao X, Fiskus W, et al. Coordinated silencing of MYC-mediated miR-29 by HDAC3 and EZH2 as a therapeutic target of histone modification in aggressive B-cell lymphomas. Cancer Cell. 2012;22:506–523.
57. Copeland RA, Keilhack H, Italiano A, et al. EZH2 inhibitor EPZ-6438 (E7438) in non-hodgkin lymphoma: Pre-clinical models and early clinical observations. Presented at the annual American Society of Hematology (ASH) meeting, 12 August 2014.
58. Mosammaparast N, Shi Y. Reversal of histone methylation: Biochemical and molecular mechanisms of histone demethylases. Annu Rev Biochem. 2010;79:155–179.

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59. Schmitges FW, Prusty AB, Faty M, et al. Histone methylation by PRC2 is inhibited by active chromatin marks. Mol Cell. 2011;42:330–341.
60. Yuan W, Xu M, Huang C, Liu N, Chen S, Zhu B. H3K36 methylation antagonizes PRC2-mediated H3K27 methylation. J Biol Chem. 2011;286:7983–7989.
61. Yuan W, Wu T, Fu H, et al. Dense chromatin activates polycomb repressive complex 2 to regulate H3 lysine 27 methylation. Science. 2012;337:971–975.
62. Barski A, Cuddapah S, Cui K, et al. High-resolution profiling of histone methylations in the human genome. Cell. 2007;129:823–837.
63. Pasini D, Malatesta M, Jung HR, et al. Characterization of an antagonistic switch between histone H3 lysine 27 methylation and acetylation in the transcriptional regulation of polycomb group target genes. Nucleic Acids Res. 2010;38:4958–4969.
64. Tie F, Banerjee R, Stratton CA, et al. CBP-mediated acetylation of histone H3 lysine 27 antagonizes Drosophila polycomb silencing. Development. 2009;136:3131–3141.
65. Kennison JA, Tamkun JW. Dosage-dependent modifiers of polycomb and antennapedia mutations in Drosophila. Proc Natl Acad Sci U S A. 1988;85:8136–8140.
66. Wilson BG, Roberts CW. SWI/SNF nucleosome remodellers and cancer. Nat Rev Cancer. 2011;11:481–492.
67. Kia SK, Gorski MM, Giannakopoulos S, Verrijzer CP. SWI/SNF mediates polycomb eviction and epigenetic reprogramming of the INK4b-ARF-INK4a locus. Mol Cell Biol. 2008;28:3457–3464.
68. Van der Vlag J, Otte AP. Transcriptional repression mediated by the human polycomb-group protein EED involves histone deacetylation. Nat Genet. 1999;23:474–478.
69. Vire E, Brenner C, Deplus R, et al. The polycomb group protein EZH2 directly controls DNA methylation. Nature. 2006;439:871–874.
70. Ohm JE, McGarvey KM, Yu X, et al. A stem cell-like chromatin pattern may predispose tumor suppressor genes to DNA hypermethylation and heritable silencing. Nat Genet. 2007;39:237–242.
71. Schlesinger Y, Straussman R, Keshet I, et al. Polycomb-mediated methylation on Lys27 of histone H3 pre-marks genes for de novo methylation in cancer. Nat Genet. 2007;39:232–236.
72. Van Haaften G, Dalgliesh GL, Davies H, et al. Somatic mutations of the histone H3K27 demethylase gene UTX in human cancer. Nat Genet. 2009;41:521–523.
73. Mar BG, Bullinger L, Basu E, et al. Sequencing histone-modifying enzymes identifies UTX mutations in acute lymphoblastic leukemia. Leukemia. 2012;26:1881–1883.
74. Oyer JA, Huang X, Zheng Y, et al. Point mutation E1099K in MMSET/NSD2 enhances its methyltranferase activity and leads to altered global chromatin methylation in lymphoid malignancies. Leukemia. 2014;28:198–201.
75. Jaffe JD, Wang Y, Chan HM, et al. Global chromatin profiling reveals NSD2 mutations in pediatric acute lymphoblastic leukemia. Nat Genet. 2013;45:1386–1391.
76. Agger K, Cloos PA, Christensen J, et al. UTX and JMJD3 are histone H3K27 demethylases involved in HOX gene regulation and development. Nature. 2007;449:731–734.
77. Lan F, Bayliss PE, Rinn JL, et al. A histone H3 lysine 27 demethylase regulates animal posterior development. Nature. 2007;449:689–694.
78. Lee MG, Villa R, Trojer P, et al. Demethylation of H3K27 regulates polycomb recruitment and H2A ubiquitination. Science. 2007;318:447–450.
79. Greenfield A, Carrel L, Pennisi D, et al. The UTX gene escapes X inactivation in mice and humans. Hum Mol Genet. 1998;7:737–742.
80. Van der Meulen J, Sanghvi V, Mavrakis K, et al. The H3K27me3 demethylase UTX is a gender-specific tumor suppressor in T-cell acute lymphoblastic leukemia. Blood. 2015;125:13–21.
81. Ntziachristos P, Tsirigos A, Welstead GG, et al. Contrasting roles of histone 3 lysine 27 demethylases in acute lymphoblastic leukaemia. Nature. 2014;514:513–517.
82. Jankowska AM, Makishima H, Tiu RV, et al. Mutational spectrum analysis of chronic myelomonocytic leukemia includes genes associated with epigenetic regulation: UTX, EZH2, and DNMT3A. Blood. 2011;118:3932–3941.
83. Kar SA, Jankowska A, Makishima H, et al. Spliceosomal gene mutations are frequent events in the diverse mutational spectrum of chronic myelomonocytic leukemia but largely absent in juvenile myelomonocytic leukemia. Haematologica. 2013;98:107–113.
84. Ezponda T, Popovic R, Zheng Y. Loss of the histone demethylase UTX contributes to multiple myeloma and sensitizes cells to EZH2 inhibitors. Blood. 2014;124(21):611. Available at. http://www.bloodjournal.org/content/124/21/611.abstract. [Accessed December 6, 2014].
85. De la Serna IL, Ohkawa Y, Imbalzano AN. Chromatin remodelling in mammalian differentiation: Lessons from ATP-dependent remodellers. Nat Rev Genet. 2006;7:461–473.
86. Kadoch C, Hargreaves DC, Hodges C, et al. Proteomic and bioinformatic analysis of mammalian SWI/SNF complexes identifies extensive roles in human malignancy. Nat Genet. 2013;45:592–601.
87. Shain AH, Pollack JR. The spectrum of SWI/SNF mutations, ubiquitous in human cancers. PLoS One. 2013;8:e55119.
88. Wilson BG, Wang X, Shen X, et al. Epigenetic antagonism between polycomb and SWI/SNF complexes during oncogenic transformation. Cancer Cell. 2010;18:316–328.
89. Versteege I, Sévenet N, Lange J, et al. Truncating mutations of hSNF5/INI1 in aggressive paediatric cancer. Nature. 1998;394:203–206.
90. Biegel JA, Zhou JY, Rorke LB, Stenstrom C, Wainwright LM, Fogelgren B. Germ-line and acquired mutations of INI1 in atypical teratoid and rhabdoid tumors. Cancer Res. 1999;59:74–79.
91. Yuge M, Nagai H, Uchida T, et al. HSNF5/INI1 gene mutations in lymphoid malignancy. Cancer Genet Cytogenet. 2000;122:37–42.
92. Fillmore CM, Xu C, Desai PT, et al. EZH2 inhibition sensitizes BRG1 and EGFR mutant lung tumours to TopoII inhibitors. Nature. 2015;520:239–242.
93. Bitler BG, Aird KM, Garipov A, et al. Synthetic lethality by targeting EZH2 methyltransferase activity in ARID1A-mutated cancers. Nat Med. 2015;21:231–238.
94. Dou Y, Hess JL. Mechanisms of transcriptional regulation by MLL and its disruption in acute leukemia. Int J Hematol. 2008;87:10–18.
95. Krivtsov AV, Armstrong SA. MLL translocations, histone modifications and leukaemia stem-cell development. Nat Rev Cancer. 2007;7:823–833.
96. Slany RK. The molecular biology of mixed lineage leukemia. Haematologica. 2009;94:984–993.
97. Thiel AT, Feng Z, Pant DK, et al. The trithorax protein partner menin acts in tandem with EZH2 to suppress C/EBPalpha and differentiation in MLL-AF9 leukemia. Haematologica. 2013;98:918–927.
98. Tanaka S, Miyagi S, Sashida G, et al. Ezh2 augments leukemogenicity by reinforcing differentiation blockage in acute myeloid leukemia. Blood. 2012;120:1107–1117.
99. Kim W, Bird GH, Neff T, et al. Targeted disruption of the EZH2–EED complex inhibits EZH2-dependent cancer. Nat Chem Biol. 2013;9:643–650.
100. Wagner EJ, Carpenter PB. Understanding the language of Lys36 methylation at histone H3. Nat Rev Mol Cell Biol. 2012;13:115–126.
101. Keats JJ, Reiman T, Belch AR, Pilarski LM. Ten years and counting: so what do we know about t(4;14)(p16;q32) multiple myeloma. Leuk Lymphoma. 2006;47:2289–2300.
102. Keats JJ, Reiman T, Maxwell CA, et al. In multiple myeloma, t(4;14)(p16;q32) is an adverse prognostic factor irrespective of FGFR3 expression. Blood. 2003;101:1520–1529.

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103. Santra M, Zhan F, Tian E, Barlogie B, Shaughnessy J Jr. A subset of multiple myeloma harboring the t(4;14)(p16;q32) translocation lacks FGFR3 expression but maintains an IGH/MMSET fusion transcript. Blood. 2003;101:2374–2376.
104. Avet-Loiseau H, Facon T, Grosbois B, et al. Oncogenesis of multiple myeloma: 14q32 and 13q chromosomal abnormalities are not randomly distributed, but correlate with natural history, immunological features, and clinical presentation. Blood. 2002;99:2185–2191.
105. Fonseca R, Blood E, Rue M, et al. Clinical and biologic implications of recurrent genomic aberrations in myeloma. Blood. 2003;101:4569–4575.
106. Popovic R, Martinez-Garcia E, Giannopoulou EG, Zhang QW, Zhang QY, Exponda T. Histone methyltransferase MMSET/NSD2 alters EZH2 binding and reprograms the myeloma epigenome through global and focal changes in H3K36 and H3K27 methylation. PLoS Genet. 2014;10:e1004566.
107. Zheng Y, Sweet SM, Popovic R, et al. Total kinetic analysis reveals how combinatorial methylation patterns are established on lysines 27 and 36 of histone H3. Proc Natl Acad Sci U S A. 2012;109:13549–13554.
108. Haber DA, Buckler AJ, Glaser T, et al. An internal deletion within an 11p13 zinc finger gene contributes to the development of Wilms' tumor. Cell. 1990;61:1257–1269.
109. Huff V. Wilms' tumours: About tumour suppressor genes, an oncogene and a chameleon gene. Nat Rev Cancer. 2011;11:111–121.
110. King-Underwood L, Pritchard-Jones K. Wilms' tumor (WT1) gene mutations occur mainly in acute myeloid leukemia and may confer drug resistance. Blood. 1998;91:2961–2968.
111. Virappane P, Gale R, Hills R, et al. Mutation of the Wilms' tumor 1 gene is a poor prognostic factor associated with chemotherapy resistance in normal karyotype acute myeloid leukemia: The United Kingdom Medical Research Council Adult Leukaemia Working Party. J Clin Oncol. 2008;26:5429–5435.
112. Wang Y, Xiao M, Chen X, et al. WT1 recruits TET2 to regulate Its target gene expression and suppress leukemia cell proliferation. Mol Cell. 2015;57:662–673.
113. Rampal R, Alkalin A, Madzo J, et al. DNA hydroxymethylation profiling reveals that WT1 mutations result in loss of TET2 function in acute myeloid leukemia. Cell Rep. 2014;9:1841–1855.
114. Sinha S, Thomas D, Yu L, et al. Mutant WT1 is associated with DNA hypermethylation of PRC2 targets in AML and responds to EZH2 inhibition. Blood. 2015;125:316–326.
115. Ernst T, Chase AJ, Score J, et al. Inactivating mutations of the histone methyltransferase gene EZH2 in myeloid disorders. Nat Genet. 2010;42:722–726.
116. Nikoloski G, Langemeijer SMC, Kuiper RP, et al. Somatic mutations of the histone methyltransferase gene EZH2 in myelodysplastic syndromes. Nat Genet. 2010;42:665–667.
117. Score J, Hidalgo-Curtis C, Jones AV, et al. Inactivation of polycomb repressive complex 2 components in myeloproliferative and myelodysplastic/myeloproliferative neoplasms. Blood. 2012;119:1208–1213.
118. Puda A, Milosevic JD, Berg T, et al. Frequent deletions of JARID2 in leukemic transformation of chronic myeloid malignancies. Am J Hematol. 2012;87:245–250.
119. Abdel-Wahab O, Adli M, LaFave LM, et al. ASXL1 mutations promote myeloid transformation through loss of PRC2-mediated gene repression. Cancer Cell. 2012;22:180–193.
120. Zhang J, Ding L, Holmfeldt L, et al. The genetic basis of early T-cell precursor acute lymphoblastic leukaemia. Nature. 2012;481:157–163.
121. Ntziachristos P, Tsirigos A, van Vlierberghe P, et al. Genetic inactivation of the polycomb repressive complex 2 in T cell acute lymphoblastic leukemia. Nat Med. 2012;18:298–301.
122. Simon C, Chagraoui J, Krost J, et al. A key role for EZH2 and associated genes in mouse and human adult T-cell acute leukemia. Genes Dev. 2012;26:651–656.
123. Baude A, Lindroth AM, Plass C. PRC2 loss amplifies Ras signaling in cancer. Nat Genet. 2014;46:1154–1155.
124. Lee W, Teckie S, Wiesner T, et al. PRC2 is recurrently inactivated through EED or SUZ12 loss in malignant peripheral nerve sheath tumors. Nat Genet. 2014;46:1227–1232.
125. Zhang M, Wang Y, Jones S, et al. Somatic mutations of SUZ12 in malignant peripheral nerve sheath tumors. Nat Genet. 2014;46:1170–1172.
126. De Raedt T, Beert E, Pasmant E, et al. PRC2 loss amplifies Ras-driven transcription and confers sensitivity to BRD4-based therapies. Nature. 2014;514:247–251.
127. Woods BA, Levine RL. The role of mutations in epigenetic regulators in myeloid malignancies. Immunol Rev. 2015;263:22–35.
128. Muto T, Santida G, Oshima M, et al. Concurrent loss of Ezh2 and Tet2 cooperates in the pathogenesis of myelodysplastic disorders. J Exp Med. 2013;210:2627–2639.
129. Bardot ES, Valdes VJ, Zhang J, et al. Polycomb subunits Ezh1 and Ezh2 regulate the Merkel cell differentiation program in skin stem cells. EMBO J. 2013;32:1990–2000.
130. Pal B, Boouras T, Shi W, et al. Global changes in the mammary epigenome are induced by hormonal cues and coordinated by Ezh2. Cell Rep. 2013;3:411–426.
131. Knutson SK, Warholic NM, Johnston LD, et al. Synergistic antitumor activity of EZH2 inhibitors and glucocorticoid receptor agonists in models of germinal center non-Hodgkin lymphomas. PLoS One. 2014;9:e111840.
132. Weisberg E, Manley PW, Coean-Jacob SW, Hochhaus A, Griffin JD. Second generation inhibitors of BCR-ABL for the treatment of imatinib-resistant chronic myeloid leukaemia. Nat Rev Cancer. 2007;7:345–356.
133. Lito P, Rosen N, Solit DB. Tumor adaptation and resistance to RAF inhibitors. Nat Med. 2013;19:1401–1409.
134. Gibaja V, Shen F, Haran J, et al. Development of secondary mutations in wild-type and mutant EZH2 alleles cooperates to confer resistance to EZH2 inhibitors. Oncogene. 2015; Article in press.
135. Lee ST, Li Z, Wu Z, et al. Context-specific regulation of NF-kappaB target gene expression by EZH2 in breast cancers. Mol Cell. 2011;43:798–810.
136. Jung HY, Jun S, Lee M, et al. PAF and EZH2 induce Wnt/beta-catenin signaling hyperactivation. Mol Cell. 2013;52:193–205.
137. Xu K, Wu ZJ, Groner AC, et al. EZH2 oncogenic activity in castration-resistant prostate cancer cells is polycomb-independent. Science. 2012;338:1465–1469.
138. He A, Shen X, Ma Q, et al. PRC2 directly methylates GATA4 and represses its transcriptional activity. Genes Dev. 2012;26:37–42.
139. Xu J, Shao Z, Li D, et al. Developmental control of polycomb subunit composition by GATA factors mediates a switch to non-canonical functions. Mol Cell. 2015;57:304–316.
140. Konski A, Weiss C, Rosier R, et al. The use of postoperative irradiation for the prevention of heterotopic bone after total hip replacement with biologic fixation (porous coated) prosthesis: An animal model. Int J Radiat Oncol Biol Phys. 1990;18:861–865.
141. Pasqualucci L, Dominguez-Sola D, Chiarenza A, et al. Inactivating mutations of acetyltransferase genes in B-cell lymphoma. Nature. 2011;471:189–195.